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(54) Title: DIFFERENTIAL LIGAND ACTIVATION OF ESTROGEN RECEPTORS ER α AND ER β AT AP1 SITES (57) Abstract <p>This invention provides methods of screening test compounds for the ability to activate or inhibit estrogen receptor β (ERβ) mediated gene activation at an AP1 site. In particular, the methods involve providing a cell comprising an estrogen receptor β (ERβ), AP1 proteins, and a construct comprising a promoter comprising an AP1 site which regulates expression of a first reporter gene. The cell is contacted with the test compound and changes in expression levels of the reporter gene are detected indicating whether the test compounds activate transcription, inactivate transcription or have no effect at the AP1 site.</p>		

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DIFFERENTIAL LIGAND ACTIVATION OF ESTROGEN
RECEPTORS ER α AND ER β AT AP1 SITES

CROSS-REFERENCE TO RELATED INVENTIONS

5

[Not Applicable]

This invention was made with the Government support under Grant No. GM 50872, awarded by the National Institutes of Health. The Government of the United States of America may have certain rights in this invention.

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BACKGROUND OF THE INVENTION

Estrogens, antiestrogens, and other nuclear transcription factor ligands are used in a wide variety of therapeutic contexts. Thus, for example, estrogens are used in the treatment of osteoporosis and other aspects (*e.g.*, vasomotor instability) of menopause, in the treatment of hypoestrogenism, and in the regulation of fertility. 15 Antiestrogens are used in the treatment of cancer. Tamoxifen, for example, is an antiestrogen that is used in breast cancer chemotherapy and is believed to function as an antitumor agent by inhibiting the action of the estrogen receptor (ER) in breast tissue (*see, e.g.*, (Sutherland *et al.* (1987) *Cancer Treat. Revs.*, 15: 183-194). Glucocorticoids are used in the treatment of pure red cell anemia, acute renal failure due to acute 20 glomerulonephritis or vasculitis, lymphocytic leukemias, lymphomas, and other conditions. Progestins or progestational agents such as medroxyprogesterone or megestrol acetate are used in the treatment of endometrial carcinoma and breast carcinoma, and are used in the regulation of fertility.

It has long been known that nuclear transcription factor ligands may have 25 profound and contradictory effects upon patients depending on physiological context. For example, estrogen and estrogen agonists may have beneficial effects, such as preventing osteoporosis and reducing serum cholesterol (Love, *et al.* (1992) *New Eng. J. Med.* 326: 852-856; Love, *et al.* (1990) *J. Natl. Cancer Inst.* 82: 1327-1332). Conversely, agonistic activity may also be harmful. Tamoxifen for example sometimes increases endometrial 30 tumor incidence (Iino *et al.* (1991) *Cancer Treat. & Res.* 53: 228-237) or switches from

0 inhibition to stimulation of estrogen dependent growth in breast tumor progression
(Parker (1992), *Cancer Surveys 14: Growth Regulation by Nuclear Hormone Receptors*.
Cold Spring Harbor Laboratory Press).

The related benzothiophene analog raloxifene (Figure 1A) has been
reported to retain the antiestrogen properties of tamoxifen in breast tissue and to show
5 minimal estrogen effects in the uterus; in addition, it has potentially beneficial estrogen-
like effects (in nonreproductive tissue such as bone and cardiovascular tissue (Jones *et al.*
(1984) *J. Med. Chem.*, 27: 1057-1066; Black *et al.* (1994) *J. Clin. Invest.*, 93: 63-69;
Sato *et al.* (1996) *FASEB J.*, 10: 905-912; Yang *et al.* (1996) *Endocrinol.*, 137: 2075-
2084; Yang *et al.*, (1996) *Science*, 273: 1222-1225).

10 One explanation for these tissue-specific actions of antiestrogens is that the
ligand-bound ER has different transactivation properties when bound to different types
of DNA enhancer elements. The estrogen receptor (ER) has been shown to mediate gene
transcription both from the classical estrogen response element (ERE) and from an AP1
enhancer element that requires ligand and the AP1 transcription factors Fos and Jun for
15 transcriptional activation (Fig. 1B). In transactivation experiments, tamoxifen inhibits the
transcription of genes that are regulated by a classical ERE, but like the natural estrogen
hormone 17 β -estradiol [E₂ (Fig. 1A)], tamoxifen activates the transcription of genes that
are under the control of an AP1 element (Webb, *et al* (1995) *Mol. Endo.*, 9: 443-456).

At the end of 1995, a second ER (ER β) was cloned from a rat prostate
20 cDNA library (Kuiper *et al.* (1996) *Proc. Natl. Acad. Sci., USA*, 93: 5925-5930). The
human (Mosselman *et al.* (1996) *FEBS Lett.*, 392: 49-53) and mouse (Tremblay *et al.*
(1997) *Mol. Endocrinol.*, 11: 353-365) homologs have also been cloned. The first
identified ER has been renamed ER α (Kuiper *et al.* (1996) *supra.*). The existence of two
ERs was postulated to present a potential new mechanism tissue-specific estrogen
25 regulation.

From the foregoing, it is clear that the activity and regulation of nuclear
transcription factor ligands, especially estrogens, is complex and the use of various
transcription factor ligands can lead to contradictory and often adverse consequences.
Thus, when electing to use a nuclear transcription factor ligand in a therapeutic context,
30 it is desirable to elucidate as precisely as possible the various modes of action (biological

0 activities) of the agent(s) under consideration. Similarly, it has long been known that various environmental compounds have estrogenic and possibly antiestrogenic activity. When evaluating the impact of such environmental estrogens and/or antiestrogens, it is desirable to evaluate their effect on all metabolic pathways in which they might prove active.

5 SUMMARY OF THE INVENTION

The present invention provides methods to rapidly and effectively screen compounds for their ability to activate or inactivate gene transcription in a previously unknown regulatory pathway: an estrogen receptor beta (ER β)-mediated AP1 pathway. This invention is premised, in part, on the surprising discovery that ER β is capable of
10 interacting with AP1 to induce transcription of a gene under AP1 control. Even more surprising was the discovery that ER β -mediated AP1 interactions can produce results significantly different than ER α -mediated AP1 interactions. For example, estradiol, which activates gene expression through an ER α -mediated AP1 interaction, actually inhibits gene activation through an ER β -mediated AP1 interaction.

15 In one embodiment, this invention provides methods of screening test compounds for differential ER α -mediated and ER β -mediated activation at an AP1 site. The methods typically involve providing a first cell comprising an estrogen receptor β (ER β), an AP1 protein, and a construct comprising a promoter comprising an AP1 site which regulates expression of a first reporter gene. The first cell is contacted with the test
20 compound and the expression of the first reporter gene is compared with ER α -mediated expression of a gene at an AP1 site in response to the same test compound. The cell can contain a heterologous estrogen receptor beta (ER β) and preferred ER β s comprise an amino acid sequence of SEQ ID NO: 3 or SEQ ID NO: 5. The cell can also contain a heterologous AP1 protein. Preferred reporter genes used in this assay include
25 chloramphenicol acetyl transferase (CAT), luciferase, β -galactosidase (β -gal), alkaline phosphatase, horse radish peroxidase (HRP), growth hormone (GH), and green fluorescent protein (GFP) with a luciferase gene or a green fluorescent protein (gene) being preferred. The test compound can be a compound known or suspected to have anti-estrogenic activity. The method can be one in which the ER α -mediated expression of a
30 gene at an AP1 site is determined by providing a second cell comprising an estrogen

0 receptor α (ER α), AP1 proteins, and a construct comprising a promoter comprising an AP1 site which regulates expression of a second reporter gene. The second cell is contacted with the test compound; and expression of the second reporter gene is detected. One preferred standard estrogen response element is from the *Xenopus* vitellogenin A2 gene. The second reporter gene and the first reporter gene can be the same species of
5 reporter gene. The cell and the second cell are the same cell.

In one embodiment, this invention provides methods screening a test compound for the ability to activate or inhibit estrogen receptor beta (ER β) mediated gene activation at an AP1 site. The methods typically involve providing a first cell comprising an estrogen receptor β (ER β), AP1 proteins, and a construct comprising a promoter
10 comprising an AP1 site which regulates expression of a first reporter gene. The cell is contacted with a test compound and expression of the first reporter gene is detected. The cell can contain a native or heterologous estrogen receptor beta (ER β). In a preferred embodiment, the ER β the amino acid sequence of Sequence ID No: 3 or Sequence ID No: 5. The first cell can also contain a heterologous AP1 protein (*e.g.*, jun and/or fos).
15 Virtually any reporter gene may be used. Preferred reporter genes include, but are not limited to chloramphenicol acetyl transferase (CAT), luciferase, β -galactosidase (β -gal), alkaline phosphatase, horse radish peroxidase (HRP), or green fluorescent protein (GFP) with a luciferase or a green fluorescent protein (GFP) being most preferred. Virtually any compound can be screened according to the methods of this invention. However,
20 preferred test compounds are compounds known to have anti-estrogenic activity.

In another embodiment, the above method can further involve providing a second cell comprising an estrogen receptor α (ER α), AP1 proteins, and a construct comprising a promoter comprising an AP1 site which regulates expression of a second reporter gene. The second cell is contacted with the test compound and the expression
25 of the second reporter gene is then detected. In addition, or alternatively, the above method can involve providing a third cell comprising an estrogen receptor α (ER α), and a construct comprising a promoter comprising a standard estrogen response element (ERE) which regulates expression of a third reporter gene. The third cell is contacted with the test compound; and expression of the third reporter gene is then detected. One
30 standard estrogen response element can be from the *Xenopus* vitellogenin A2 gene.

0 Additionally or alternatively, the above method can also involve providing a fourth cell comprising an estrogen receptor β (ER β), and a construct comprising a promoter comprising a standard estrogen response element (ERE) which regulates expression of a fourth reporter gene. The fourth cell is contacted with the test compound and expression of the fourth reporter gene is detected. Again the standard estrogen response element can
5 be from the *Xenopus* vitellogenin A2 gene. In one embodiment, the first cell and said third cell are the same cell, while in another embodiment, the first cell and said fourth cell are the same cell.

Any of the above-described assays can be run to detect or identify inhibitors that block compounds that activate ER β -mediated AP1 gene transcription. This
10 typically involves performing the assays as described above, but, in addition, contacting the first cell with a second compound, in addition to the test compound, wherein said second compound is known to activate transcription through estrogen receptor β (ER β) mediated gene activation at an AP1 site. Detecting then comprises detecting test compound mediated decrease in said estrogen receptor β (ER β) mediated gene activation
15 at an AP1 site. In a particularly preferred embodiment, the detecting can involve comparing the expression of the first reporter gene in the presence of the test compound and the second compound with the expression of the reporter gene in the presence of the second compound without the test compound.

In one embodiment, the second compound known to activate transcription
20 through estrogen receptor β (ER β) mediated gene activation at an AP1 site is identified by a method involving providing a second cell comprising an estrogen receptor β (ER β), and AP1 protein, and a construct comprising a promoter comprising an AP1 site that regulates expression of a second reporter gene. The second cell is contacted with the second compound and the expression of the second reporter gene is detected where an
25 increase in expression of the second reporter gene produced by the compound indicates that said second compound activates transcription through ER β at an AP1 site.

The assays of this invention can also be used to detect or identify inhibitors that block compounds that inhibit ER β -mediated AP1 gene transcription. These methods involve performing the assays as described above, while additionally contacting the first
30 cell with a second compound, in addition to the test compound, where the second

0 compound is known to inhibit transcription through estrogen receptor β (ER β) mediated activity at an AP1 site. Expression of the reporter gene is detected where the detection comprises detecting test compound mediated increase in estrogen receptor β (ER β) mediated gene activation at an AP1 site. The detecting can involve comparing expression of the first reporter gene in the presence of both the second compound and the test
5 compound with expression of the first reporter gene in the presence of the second compound without the test compound.

The second compound known to inhibit transcription through estrogen receptor β (ER β) mediated gene activation at an AP1 site can be identified by providing a second cell comprising an estrogen receptor β (ER β), and AP1 protein, and a construct
10 comprising a promoter comprising an AP1 site that regulates expression of a second reporter gene. The second cell is contacted with the second compound; and expression of the second reporter gene is detected. A decrease in expression of said second reporter gene produced by the second compound indicates that the second compound inhibits transcription through ER β at the AP1 site.

15 This invention also provides for any of the cells described above or herein. In one embodiment the cell comprises an estrogen receptor β (ER β), an AP1 protein (*e.g.*, jun or fos), and a construct comprising a promoter comprising an AP1 site which regulates expression of a first reporter gene. The cell can additionally include a receptor for a nuclear transcription factor ligand preferably for a nuclear transcription factor ligand
20 other than estrogen. The cell preferably contains a heterologous ER β , more preferably an ER β comprising an amino acid sequence of Sequence ID No: 3 or Sequence ID No: 5. The AP1 protein can be a native AP1 protein or a heterologous AP1 protein. The reporter gene can be one selected from the group consisting of chloramphenicol acetyl transferase (CAT), luciferase, β -galactosidase (β -gal), alkaline phosphatase, horse radish
25 peroxidase (HRP), and green fluorescent protein (GFP), but in particularly preferred embodiment, the reporter gene encodes a luciferase or a green fluorescent protein (GFP). The cell can additionally include a standard estrogen response element (ERE) which regulates expression of a second reporter gene. One preferred standard estrogen response element is from the *Xenopus* vitellogenin A2 gene. Preferred cells of this invention are
30 mammalian cells and particularly preferred cells are derived from breast tissue or from

0 uterine tissue. The cells may be neoplastic cells. Any of the above-described assays can be run to detect or identify inhibitors that block compounds that activate ER β -mediated AP1 gene transcription.

In still another embodiment, this invention provides methods of screening a nuclear transcription factor ligand for the ability to modulate estrogen receptor β mediated activation or inactivation of transcription at an AP1 site. The methods involve
5 providing a first cell containing an estrogen receptor β (ER β), an AP1 protein, a receptor for the nuclear transcription factor ligand, and a construct comprising a promoter comprising an AP1 site which regulates expression of a first reporter gene. The cell is contacted with the transcription factor ligand and with a compound having ER β mediated
10 activity at the AP1 site. Expression of the first reporter gene is then detected.

The method can further involve providing a second cell containing an estrogen receptor β (ER β), a receptor for the nuclear transcription factor ligand, and a construct comprising a promoter comprising an estrogen response element (ERE) that regulates expression of a second reporter gene. The second cell is contacted with the
15 transcription factor ligand and with the compound having AP-1 mediated estrogenic activity and expression of the second reporter gene is detected. The first and second cells can be the same or different.

Alternatively, or in addition, the method can further involve providing a second cell containing a cognate receptor of the transcription factor ligand, and a
20 promoter comprising a response element for the cognate receptor that regulates expression of a second reporter gene. The second cell is contacted with the transcription factor ligand and with the compound having compound having ER β mediated activity at said AP1 site expression of the second reporter gene is detected. Again, the first and second cells can be the same or different cells.

25 In any of the above-described methods the nuclear transcription factor ligand can be selected from the group consisting of a glucocorticoid, a progestin, vitamin D, retinoic acid, a an androgen, a mineralcorticoid, and a prostaglandin. Similarly, the cognate receptor can be selected from the group consisting of an estrogen receptor, a glucocorticoid receptor, a progestin PR-A receptor, and progestin PR-B receptor,
30 androgen receptor, a mineralcorticoid receptor, and a prostaglandin receptor. In a

0 particularly preferred embodiment, the ER β comprises an amino acid sequence of Figure 5 or Figure 6A. The ER β can be a heterologous ER β . Similarly, the receptor for the nuclear transcription factor ligand can be heterologous to the cell. The cell can express an AP1 protein (*e.g.*, jun or fos) from a heterologous DNA. In one particularly preferred embodiment, the nuclear transcription factor is a progestin; and said receptor for the
5 nuclear transcription factor ligand is a progestin receptor. In another preferred embodiment, the nuclear transcription factor is a glucocorticoid and said receptor for said nuclear transcription factor ligand is a GR receptor.

This invention also provides methods of screening an agent for the ability to alter modulation of estrogen receptor β (ER β) activation or inactivation of
10 transcription at an AP1 site by a nuclear transcription factor ligand. The methods involve providing a first cell containing an estrogen receptor β (ER β), an AP1 protein, a receptor for the nuclear transcription factor ligand, and a promoter comprising an AP1 site which regulates expression of a first reporter gene. The first cell is contacted with the transcription factor ligand, with a compound having ER β mediated activity at an AP1 site,
15 and with the agent and expression of the first reporter gene is detected.

This method can further involve providing a second cell containing an estrogen receptor β (ER β), a receptor for the nuclear transcription factor ligand, and a promoter comprising an estrogen response element (ERE) that regulates expression of a
20 second reporter gene. The second cell is contacted with the transcription factor ligand and with the compound having AP-1 mediated estrogenic activity and expression of the reporter gene is detected. The first and second cell can be the same cell or different cells. The nuclear transcription factor can be one selected from the group consisting of a glucocorticoid, a progestin, vitamin D, retinoic acid, an androgen, a mineralcorticoid, a
25 prostaglandin. Similarly, the nuclear transcription factor ligand is selected from the group consisting of an estrogen receptor, a glucocorticoid receptor, a progestin PR-A receptor, progestin PR-B receptor, an androgen receptor, a mineralcorticoid receptor, and a prostaglandin receptor. Again, in any of the assays described herein, the ER β can be a heterologous ER β and in a preferred embodiment, the ER β comprises an amino acid
30 sequence of Sequence ID No: 3 or Sequence ID No: 5 or is encoded by a nucleic acid

0 sequence of Sequence ID No: 3 or Sequence ID No: 6. The AP1 protein(s) and/or the
receptor for the nuclear transcription factor ligand can also be native to the cell or
heterologous. In one particularly preferred embodiment, the nuclear transcription factor
is a progestin; and the receptor for said nuclear transcription factor ligand is a progestin
receptor, while in another preferred embodiment, the nuclear transcription factor is a
5 glucocorticoid and the receptor for said nuclear transcription factor ligand is a GR
receptor.

This invention also provides kits for screening a compound for the ability
to activate or inhibit estrogen receptor β (ER β) mediated gene activation at an AP1 site.
The kits can include a container containing a cell comprising an estrogen receptor β
10 (ER β), an AP1 protein (*e.g.*, jun and/or fos), and a construct comprising a promoter
comprising an AP1 site which regulates expression of a first reporter gene. The cell of the
kits can further a receptor for a nuclear transcription factor ligand, preferably a nuclear
transcription factor ligand other than estrogen. The kits can also further include
instructional materials containing protocols for the practice of any of the assay methods
15 described herein.

DEFINITIONS

The terms "activate transcription" or "inhibit transcription" as used herein
refer to the upregulation of transcription of a gene or the downregulation of transcription
of a gene. It will be appreciated that either complete, or partial, "turning on" or "turning
20 off" are regarded herein as activation or inhibition, respectively. Activation and
inhibition of transcription are typically measured with respect to a control or controls
where the control or controls involve a similar treatment lacking the compound or agent
in question and/or contain a standard agent (*e.g.*, E₂ or tamoxifen). It will also be
appreciated that there may exist a baseline level of transcription (*e.g.* of a particular
25 reporter gene) even where an assay cell of this invention is "unstimulated" (*e.g.* the
receptor in question is unliganded), *i.e.*, without exogenously supplied ligand). In this
case, it may be possible to see inhibition without necessarily applying exogenous activator
see, *e.g.*, Example 1).

As used herein an antiestrogen is a compound that substantially inhibits
30 estrogen activity as measured in an assay for estrogenic activity, for example, cellular

0 assays as described in Webb *et al. Mol. Endocrinol.*, 6:157-167 (1993). More generally, a "transcription factor antagonist" is a compound that substantially inhibits transcription factor activity as measured in a standard assay for that transcription factor activity.

A "nuclear transcription factor" as used herein refers to members of the nuclear transcription factor superfamily. This is a family of receptors that are capable of
5 entering the nucleus of a cell and once there, effecting the up-regulation or down-regulation of one or more genes. A "nuclear transcription factor ligand" is a compound that binds to a nuclear transcription factor. Preferred nuclear transcription factors are typically steroid receptors, however, the group is not so limited. Nuclear transcription factor ligands include, but are not limited to estrogen, progestins, androgens,
10 mineralcorticoids, glucocorticoids, retinoic acid, vitamin D, and prostaglandins. Transcription factor ligands also include analogues of naturally occurring factors and blocking agents (antagonists) of such factors. Transcription factors also include, as they are identified, the ligands that bind orphan receptors (those nuclear transcription factors which have been identified by sequence homology, but whose ligand is yet unidentified).
15 It will be appreciated that when used in the context of a modulator of estrogen activity, the nuclear transcription factor ligand is typically one other than estrogen (or other than the estrogen or estrogen agonist whose activity is being modulated). Nuclear transcription factors typically mediate their activity through binding of a cognate receptor in the cell nucleus. The term cognate receptor" refers to a receptor of the type that is typically bound by the transcription ligand in question. Thus, the cognate receptor for an
20 estrogen is an estrogen receptor, the cognate receptor for a glucocorticoid is a glucocorticoid receptor, the receptor for a progestin is a progestin receptor, and so forth. The cognate receptor includes the native (naturally occurring) form as well as modified receptors.

25 The phrase estrogen receptor beta (ER β)-mediated activation or inactivation of gene transcription at an AP1 site refers to the activation or inactivation of a gene (e.g., a reporter gene) under control of an AP1 site by the interaction of that AP1 site with a liganded ER β receptor. Similarly ER α -mediated activation or inactivation refers to gene regulation mediated by the interaction of ER α . Inactivation or inactivation
30 at an ERE refers to activation or inactivation of a gene under control of an ERE.

0 The phrase "differential ER α -mediated and ER β -mediated activation at an AP1 site" refers to differences between ER α - and ER β -mediated gene activation at an AP1 site in response to the same ligand. Differential activation can be reflected in significant differences in levels of gene activation or inactivation by the same ligand depending on whether it interacts with ER α or ER β . Differential activation can also
5 reflect differences in the "sign" of gene activation. Thus differential activation can refer to ER β -mediated activation of transcription at an AP1 site and ER α -mediated inactivation of gene transcription at an AP1 site in response to the same ligand. Conversely, differential activation can refer to ER β -mediated inactivation of transcription at an AP1 site and ER α -mediated activation of gene transcription at an AP1 site in response to the
10 same ligand.

 AP1-mediated estrogenic/agonist activity, as used herein, refers to activation of a gene under the control of an AP1 site (also referred to as an AP1 response element) mediated by the interaction of a nuclear transcription factor with the AP1 site. When used in reference to ER mediated activation of a gene controlled by the AP1 site,
15 the pathway is referred to as the indirect estrogen response (in contrast to the classical estrogen response which is mediated through an ERE). A general description of the AP1 site is found in Angel & Kann, *Biochem. Biophys. Acta.*, 1072: 129-157 (1991) and Angel, *et al.*, *Cell*, 49: 729-739 (1987).

 A "compound having AP1 mediated estrogenic activity" refers to a
20 compound that, when present in a cell containing a gene under control of an AP1 site and AP1 proteins, activates transcription of the gene under control of the AP1 site.

 A "compound having the ability to inactivate or inhibit estrogen receptor beta (ER β) mediated gene activation at an AP1 site refers to a compound that is capable of upregulating or downregulating transcription of a gene under the control of an AP1 site
25 through its interaction (*e.g.*, binding) of an ER β .

 The phrases "modulate estrogen activation" or "modulation of estrogen activation" refer to alteration of the estrogen induced expression of a particular gene. Where the phrase additionally recites "at an AP1 site or at an ERE" the phrase refers to alteration of the level of estrogen induced expression of one or more genes under control
30 of the AP1 site or ERE site respectively. The phrase "detecting expression" when used

0 with reference to a reporter gene refers to detection of presence or absence of expression of the reporter gene or to quantification of expression level of the reporter gene. The quantification can be either an absolute measurement or a relative measurement (*e.g.*, in comparison to another expressed gene).

The term "operably linked" refers to functional linkage between a nucleic acid expression control sequence (such as a promoter, signal sequence, or transcription factor binding site) and a second nucleic acid sequence, wherein the expression control sequence affects transcription and/or translation of the nucleic acid corresponding to the second sequence.

The term "recombinant" when used with reference to a cell indicates that the cell replicates a heterologous nucleic acid, or expresses a peptide or protein encoded by a heterologous nucleic acid. Recombinant cells can express genes that are not found within the native (non-recombinant) form of the cell. Recombinant cells can also express genes found in the native form of the cell wherein the genes are modified and re-introduced into the cell by artificial means. Recombinant expression refers to the expression of the heterologous nucleic acid by such a recombinant cell.

A "heterologous nucleic acid", as used herein, is one that originates from a foreign source (or species) or, if from the same source, is modified from its original form. Thus, a heterologous nucleic acid operably linked to a promoter is from a source different from that from which the promoter was derived, or, if from the same source, is modified from its original form. Modification of the heterologous sequence may occur, *e.g.*, by treating the DNA with a restriction enzyme to generate a DNA fragment that is capable of being operably linked to the promoter. Techniques such as site-directed mutagenesis are also useful for modifying a heterologous sequence. Similarly, a "heterologous protein" refers to a protein that originates from a foreign source (*e.g.*, different cell or species) or, if from the same source, is modified from its original form, or is expressed from a heterologous nucleic acid.

A "recombinant expression cassette" or simply an "expression cassette" is a nucleic acid construct, generated recombinantly or synthetically, with nucleic acid elements that are capable of effecting expression of a structural gene in hosts compatible with such sequences. Expression cassettes include at least promoters and optionally,

0 transcription termination signals. Typically, the recombinant expression cassette includes a nucleic acid to be transcribed (*e.g.*, a nucleic acid encoding a desired polypeptide), and a promoter. Additional factors necessary or helpful in effecting expression may also be used as described herein. For example, an expression cassette can also include nucleotide sequences that encode a signal sequence that directs secretion of an expressed protein
5 from the host cell.

Xenoestrogens are defined here to include any compound having estrogenic activity in the assays described herein, which is derived from a source outside the human body. Environmental compounds as used herein can be derived from a wide variety of sources including plants, soil, water, foods. They also include synthetic
10 compounds such as chlorinated organics, polycyclic aromatic hydrocarbons, herbicides, pesticides, pharmaceuticals and the like.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A illustrates the structure of five estrogen receptor (ER) ligands: Estradiol (E_2), diethylstilbestrol (DES), ICI 184,384, raloxifene (Ral), and tamoxifen
15 (Tam).

Figure 1B illustrates two estrogen receptor (ER) response elements: a simple (classical) estrogen response element (ERE) and an ER dependent AP1 element described also in USSN 08/410,807, in USSN 60/051,309, and by Webb *et al* (1995) *Mol. Endo.*, 9: 443-456.

20 Figure 2 illustrates ER β action at an estrogen response element (ERE). HeLa cells were transfected with an ERE-regulated luciferase reporter plasmid and an expression vector for rat ER β as described herein. Transfected cells were treated with the ligands (E_2 , 0.1 μ M; DES, 1 μ M, Ral, 1 μ M, tamoxifen 5 μ M; and ICI, 1 μ M) or an ethyl alcohol (EtOH) vehicle control. All assays were done with at least triplicate transfections.
25 Error bars show deviations between wells from a single representative transfection.

Figure 3 illustrates ER α action at an AP1 element. HeLa cells were transfected with an AP1 reporter plasmid and an ER α expression plasmid and treated with the five ligands (*see, e.g.*, Figure 2). Ligand concentrations were E_2 , 0.1 μ M; DES, 1 μ M; Ral, 1 μ M; Tam, 5 μ M, and ICI, 1 μ M. Error bars are as in Figure 2.

0 Figure 4 illustrates ER β activation and inhibition at AP1. (A) ER β action
at an AP1 response element. HeLa cells were transfected with an AP1 reporter plasmid
and a rat ER β expression plasmid as described herein. Transfected cells were treated with
the following ligand concentrations: E $_2$, 0.1 μ M; DES, 1 μ M; Ral, 1 μ M, Tam, 5 μ M; and
ICI, 1 μ M. (B) Dose response of raloxifene induction with ER β at an AP1 element.
5 HeLa cells transfected as described for A were treated with the indicated range of
raloxifene concentrations. (D) Comparative inhibition of raloxifene induction by E $_2$ and
DES. HeLa cells were transfected as described for (A) and treated with ligands. The left
panel shows transactivation induction by raloxifene (1 μ M), the lack of induction by E $_2$
(0.1 μ M) and induction to the amount observed with the control (no ligand added). The
10 right panel shows the dose dependence of inhibition of raloxifene (1 μ M) induction by
DES (solid line) and E $_2$ (Dashed line). (D) Raloxifene overriding E $_2$ inhibition. HeLa cells
were transfected as described for (A) and treated with ligands. The left panel shows the
transcription induction resulting from the vehicle control (EtOH), Ral (1 μ M) plus E $_2$ (10
nM), and E $_2$ (10 nM) alone. The right panel shows the dose dependence of raloxifene
15 induction in the presence of E $_2$ (10 nM).

Figure 5 illustrates ligand-dependent ER β activity in three cell types;
Ishikawa cells, MCF7 cells and MDA453 cells. (A) Ligand-dependent ER β action at an
AP1 element in Ishikawa cells. Ishikawa cells were transfected with an AP1-regulated
luciferase reporter plasmid and an ER β expression plasmid. Transfected cells were treated
20 with one or two ligands as indicated (E $_2$, 0.1 μ M; DES, 1 μ M; Ral, 1 μ M, Tam, 5 μ M;
and ICI, 1 μ M; or an EtOH vehicle (control)). (B) Ligand dependent ER β action at an
AP1 element in MCF7 cells. MCF7 cells were treated and analyzed as described for (A).
Ligand dependent ER β action at an AP1 element in MDA453 cells. MDA453 cells were
treated and analyzed as described for (A).

25 DETAILED DESCRIPTION

Antiestrogens are therapeutic agents for the treatment and possible
prevention of breast cancer. Tamoxifen (Figure 1A), for example, is an antiestrogen that
is used in breast cancer chemotherapy and is believed to function as an antitumor agent
by inhibiting the action of the estrogen receptor (ER) in breast tissue (Grainger *et al.*
30 (1996) *Nature Med.*, 2: 381-385). Paradoxically, tamoxifen appears to function as an

0 estrogen-like ligand in uterine tissue, and this tissue-specific iatrogenic effect may explain the increased risk of uterine cancer that is observed with prolonged tamoxifen therapy (Kedar *et al.* (1994) *Lancet*, 343: 1318-1321).

The related benzothiophene analog raloxifene (Fig. 1A) has been reported to retain the antiestrogen properties of tamoxifen in breast tissue and to show minimal
5 estrogen effects in the uterus; in addition, it has potentially beneficial estrogen-like effects (in nonreproductive tissue such as bone and cardiovascular tissue (Jones *et al.* (1984) *J. Med. Chem.*, 27: 1057-1066; Black *et al.* (1994) *J. Clin. Invest.*, 93: 63-69; Sato *et al.* (1996) *FASEB J.*, 10: 905-912; Yang *et al.* (1996) *Endocrinol.*, 137: 2075-2084; Yang *et al.*, (1996) *Science*, 273: 1222-1225)). One explanation for these tissue-specific actions
10 of antiestrogens is that the ligand-bound ER may have different transactivation properties when bound to different types of DNA enhancer elements.

The classical estrogen response element (ERE) is composed of two inverted hexanucleotide repeats, and ligand-bound ER binds to the ERE as a homodimer (Fig. 1B). The ER also mediates gene transcription from an AP1 enhancer element that
15 requires ligand and the AP1 transcription factors Fos and Jun for transcriptional activation (Fig. 1B) (Umayahara *et al.* (1994) *J. Biol. Chem.*, 269: 16433-16442). In transactivation experiments, tamoxifen inhibits the transcription of genes that are regulated by a classical ERE, but like the natural estrogen hormone 17 β -estradiol [E₂ (Fig. 1A)], tamoxifen activates the transcription of genes that are under the control of an AP1 element (Webb
20 *et al.* (1995) *Mol. Endocrinol.*, 9: 443-456).

At the end of 1995, a second ER (ER β) was cloned from a rat prostate cDNA library (Kuiper *et al.* (1996) *Proc. Natl. Acad. Sci. USA*, 93: 5925-5930). The human (Mosselman *et al.* (1996) *FEBS Lett.*, 392: 49-53) and mouse (Tremblay *et al.* (1997) *Mol. Endocrinol.*, 11: 353-365) homologs were also cloned. The first identified
25 ER has been renamed ER α (Kuiper *et al.* (1996) *supra.*). It was a discovery of this invention that ER β presents another source of tissue-specific estrogen regulation, particularly as mediated through the AP1 site. In particular, it was a discovery of this invention that ER α and ER β respond differently to certain ligands at an AP1 element. The results described herein suggest different regulatory functions for the two ER
30 subtypes. This invention thus provides materials and methods for screening for

0 compounds that exhibit differential activity depending on whether their activity is mediated through ER α or ER β . In addition, this invention provides materials and methods for determining whether a compound is capable of activate or inhibit estrogen receptor β (ER β) mediated gene activation (transcription) at an AP1 site.

I. Screening Methods and Compositions.

5 It was a discovery of this ER β can interact with a AP1 site to activate or inactivate expression (e.g.transcription) of a gene under the control of the AP1 site. Moreover, it was a particularly surprising discovery that putative estrogens can actually demonstrate "antiestrogenic" activity in an ER β /AP1 pathway (where antiestrogenic activity in this context is as compared to the activity of an estrogen in the classical
10 ER α /ERE pathway). Thus, where an estrogen would activate transcription in an ER α /ERE pathway the estrogen inactivates transcription in an ER α /AP1 pathway. Conversely, putative antiestrogens can demonstrate estrogenic activity in an ER β /AP1 pathway. This invention thus provides methods for detecting antiestrogenic activity of putative estrogens, or for detecting estrogenic activity of putative antiestrogens. More
15 generally, as explained below, this invention provides methods of screening compounds for the ability to activate or inhibit estrogen receptor β (ER β) mediated gene activation at an AP1 site. This allows identification of previously unsuspected environmental estrogens or antiestrogens or for screening of compounds for those that have desirable estrogenic or antiestrogenic properties. Such compounds are expected to be useful for
20 the treatment or the prevention of various cancers (e.g.breast cancer, ovarian cancer, endometrial cancer) and other diseases (e.g. endometriosis) mediated by estrogen.

A) Screening for ER β mediated AP1 activation or inhibition.

This invention provides efficient ways to screen large numbers of test compounds for the ability to activate or inhibit estrogen receptor β (ER β) mediated gene
25 activation at an AP1 site. In one embodiment, the methods utilize a cell containing an estrogen receptor beta (ER β), an AP1 protein, and a construct comprising a promoter and reporter gene under the control of an AP1 site such that ER β interaction with the AP1 site, can increase or inhibit expression (e.g., transcription) of the reporter gene. The cell is contacted with one or more compounds whose ER β activity at AP1 it is desired to
30 evaluate. In a preferred embodiment, the expression level of the reporter gene in the cell

0 contacted with the compound is compared to the expression level of a cell contacted by
a control (*e.g.*, identical culture conditions lacking the test compound and/or with a
reference compound *e.g.*, estradiol or tamoxifen). A decrease in expression level of the
reporter gene indicates that the test compound inhibits ER β -mediated expression
(transcription) at an AP1, site, while an increase in expression level of the reporter gene
5 indicates that the test compound activates ER β -mediated expression (transcription) at an
AP1 site.

The criteria used to evaluate a change in expression level of the reporter
gene in this assay, and the other assays described herein, are those standard in the art.
Thus, for example, a statistically significant difference in expression level between the test
10 and control experiments are scored as a valid change. In a preferred embodiment, the
expression level may change by a factor 1.5 or more, preferably by factor of 2 or more,
more preferably by a factor of 4 or more, and most preferably by a factor of 5 or even 10
or more.

Screening for differential ER α and ER β mediated activity.

15 It will be appreciated that using the methods of this invention, the ability
of compounds to activate or inhibit ER β -mediated transcription at an AP1 site can be
compared to the ability of those compounds to activate or inhibit ER β -mediated activity
at an ERE site or to the ability of those compounds to activate or inhibit ER α -mediated
activity at an AP1 or ERE. In this manner, compounds having a highly specific mode of
20 activity across a wide tissue distribution, or alternatively compounds having a highly
variable mode of activity can be identified.

Four preferred estrogen receptor based assays are illustrated in Table 1.
These correspond to ER α -mediated ERE activity, ER α -mediated AP1 activity, ER β -
mediated ERE activity, and ER β -mediated AP1 activity. It was a discovery of the present
25 invention that various compounds exhibit differential activity in these various assays.

0

Table 1. Illustration of estrogen receptor based assays.

	ER	ER
	α	β
ERE/reporter	Classical pathway	Classical pathway
gene	Classical pathway	Classical pathway
AP1/reporter	Indirect pathway	Indirect pathway
gene	Indirect pathway	Indirect pathway

5

This is illustrated in Table 2, where it can be seen that estrogen activates transcription in both the classical response (at an ERE) and in the indirect response (at an AP1) when the interaction is mediated by ER α . In contrast, estrogen acts as an inhibitor of transcription at AP1 when the interaction is mediated by ER β . In contrast, the estrogen antagonist tamoxifen appears to always act as an inhibitor at an ERE, but an activator of transcription at an AP1 site. Moreover, the activity of ER β does not appear to be tissue restricted.

15

0 **Table 2.** Illustration of the activity of estradiol (E₂) and an estrogen antagonist (tamoxifen) in each of the ER assays.

		ER	
		α	β
5	ERE/reporter gene	Ac	Ac
	Estradiol	tivates	tivates
	Tamoxifen	Inh ibits	Inh ibits
10	AP1/reporter gene	Ac	Inh
	Estradiol	tivates	ibits
	Tamoxifen	Ac tivates	Ac tivates

15 The assay for ER β -mediated AP1 activity is described above. The remaining assays are performed in an analogous manner. Thus, the ER α -mediated activity assays simply involve substituting ER α for ER β , and the ERE activity assays simply involve substituting the ERE/reporter gene construct for the AP1/reporter gene construct. The ER α assays (both for ERE and AP1 activity) are described in detail in USSN 08/410,807, in USSN 60/051,309, and by Webb *et al* (1995) *Mol. Endo.*, 9: 443-456).

20 The assay for ER β -mediated ERE activity utilizes a cell containing an estrogen receptor beta (ER β), and a construct comprising a promoter and reporter gene under the control of an ERE site such that ER β interaction with the ERE site, can increase or inhibit expression (*e.g.*, transcription) of the reporter gene. The cell is contacted with one or more compounds whose ER β activity at an ERE it is desired to evaluate. In a preferred embodiment, the expression level of the reporter gene in the cell contacted with
25 the compound is compared to the expression level of a cell contacted by a control (*e.g.*, identical culture conditions lacking the test compound and/or with a reference compound *e.g.*, estradiol or tamoxifen). A decrease in expression level of the reporter gene indicates that the test compound inhibits ER β -mediated expression (transcription) at an ERE, while

0 an increase in expression level of the reporter gene indicates that the test compound activates ER β -mediated expression (transcription) at an ERE site.

While, in a preferred embodiment, each assay is performed in a separate cell, it will be appreciated that AP1 and ERE assays can be combined and performed in a single cell. In this case, the AP1/reporter gene construct preferably utilizes a different
5 reporter gene than the ERE/reporter gene construct so that AP1 activation or inactivation can be distinguished from ERE activation or inactivation.

Screening for inhibitor activity.

The above-describe assays can also be used to identify (screen for) compounds that inhibit other compounds which have ER α -mediated or ER β -mediated
10 activity an ERE or at an AP-1 site. These assays are performed in the same manner as the assays described above. In this instance, however, the cell is contacted with two compounds, a test compound that is being screened for inhibitory activity and a second compound for which an inhibitor (or alternatively an agonist) is sought.

Thus, for example, where it is desired to identify a test compound having
15 ER β -mediated estrogen inhibitory activity at an AP1 site, the cell containing ER β , an AP1 protein, and a reporter gene under control of an AP1 site is contacted with estrogen and the test compound. If the compound inhibits the characteristic ER β -mediated estrogen activity at AP1, the compound is an inhibitor. It should be noted that in this case, ER β -mediated estrogen activity at AP1 inhibits transcription, thus an estrogen inhibitor in this
20 context actually increases ER β -mediated transcription at AP1. This is illustrated in Example 1, where it is shown that tamoxifen is one such inhibitor.

Inhibitors, or agonists, of ER β -mediated or ER α -mediated estrogenic or antiestrogenic activity at ERE and at AP1 can be screened in an analogous manner.

D) Screening for environmental estrogens or antiestrogens.

25 As indicated above, this invention allows for screening of test compounds for estrogenic or antiestrogenic activity mediated through ER β or ER α at an ERE or at an AP1 site. The assays are particularly useful for screening environmental compounds for estrogenic or antiestrogenic activity. Environmental compounds having estrogenic activity are referred to here as xenoestrogens. Xenoestrogens include any compound
30 derived from a source outside the human body, having estrogenic activity in the assays

0 described herein. Environmental compounds as used herein can be derived from a wide variety of sources including plants, soil, water, foods. They also include synthetic compounds such as chlorinated organics, polycyclic aromatic hydrocarbons, herbicides, pesticides, pharmaceuticals and the like.

It will be appreciated that environmental estrogens often are only weakly
5 active. Consequently, particularly when testing an environmental compound for estrogenic or antiestrogenic activity, it is often desirably to maximize sensitivity of the assay. This may be accomplished by using cells that produce the methods typically comprise cultured cells that produce high levels of the human estrogen receptor ($ER\alpha$ or $ER\beta$). Such cells include, but are not limited to MCF-7 cells (ATCC No. HTB 22), MDA453 cells (ATCC
10 No. HTB 131), ZR-75-1 cells (ATCC No. CRL 1500) or ERC1 cells described in Kushner *et al.* (1990) *Mol. Endocrinol.*, 4:1465-1473, and ERC2 and ERC3 cells as described by Webb *et al.* (1993) *Mol. Endocrinol.*, 6:157-167.

It is also known that environmental estrogens may show synergistic activity in combination. Thus, in one embodiment, two or more suspected environmental
15 estrogens are assayed according to the above methods in combination. It will be recognized, however, that such combined testing is not limited simply to environmental estrogens but rather, any combination of agents can be screened simultaneously.

Screening for transcription factor modulation of $ER\beta$ activity at AP1.

It has been demonstrated that various nuclear transcription factors (*e.g.*,
20 progesterone, glucocorticoids, *etc.*) interact with the $ER\alpha$ -mediated estrogenic activity at the AP1 site (*see, e.g.*, USSN 60/051,309). It is believed that $ER\beta$ is also capable of such interactions at AP1. Thus, in another embodiment, this invention provides assays (methods of screening) nuclear transcription factor ligands, and putative or known transcription factor ligand agonists or antagonists for the ability to modulate $ER\beta$ -
25 mediated activation or inactivation of transcription at an AP1 site.

These assays are performed in the same manner as the assays described above, however the assay cell additionally contains a receptor for a second nuclear transcription ligand (preferably a ligand other than estrogen). Thus, the cell contains an estrogen receptor beta ($ER\beta$), an AP1 protein, a receptor for a second nuclear
30 transcription factor ligand, and a construct comprising a promoter comprising an AP1 site

0 which regulates expression of a reporter gene. The cell is contacted with both a transcription factor ligand that is to be screened and with a compound having ER β mediated activity at an AP1 site.

Alteration of the typical activity (level of AP1 regulated reporter gene expression) of the compound having ER β -mediated activity at an AP1 site by the presence
5 of the compound being screened (the test transcription factor ligand) indicates that the screened compound is capable of modulating an ER β -mediated AP1 response of the compound having ER β -mediated activity at an AP1 site. Preferred second nuclear transcription factor ligands include, but are not limited to glucocorticoids, progestins, vitamin D, retinoic acid, androgens, mineralcorticoids, and prostaglandins.

10 Similarly, inhibitors, or agonists, of the test compound can be screened by running the same assay in the presence of the inhibitor that is to be screened.

II. Cell Types

The assay methods of this invention provide methods for evaluating the ability of a test, or control, compound to activate or inhibit transcription through
15 interaction with a transcription factor receptor (*e.g.*, estrogen receptor). Thus, in a preferred embodiment, the cells used in the assays of this invention preferably contain at least one transcription factor receptor.

For example, where it is desired to screen for activity of a compound mediated by the estrogen receptor α (ER α) cells are preferably provided that contain ER α
20 and where it is desired to screen for activity of a compound mediated by estrogen receptor β (ER β) cells are preferably provided that contain ER β .

Where it is desired to screen for the ability of a nuclear transcription factor ligand modulate estrogen receptor (α or β) mediated activation or inactivation of transcription at an AP1 site, the cell preferably include, in addition to the particular ER α
25 or ER β at least a second nuclear transcription factor receptor (*e.g.*, glucocorticoid receptor (GR)). Cells that naturally express one or more of the desired receptor types can be used in the assays of this invention. Alternatively, cells can be modified (*e.g.*, through recombinant DNA techniques) to express ER α and/or ER β and/or the transcription factor receptor of choice.

0 Suitable cells for practicing the methods of this invention include, but are not limited to cells derived from a uterine cervical adenocarcinoma (HeLa) , a hypothalamic cell line (GT1-1 (Mellon *et al.* (1990) *Neuron*, 5: 1-10), MCF-7 cells (ATCC No. HTB 22), MDA453 cells (ATCC No. HTB 131), ZR-75-1 cells (ATCC No. CRL 1500) or ERC1 cells described in Kushner *et al.*, *Mol. Endocrinol.*, 4:1465-1473
5 (1990). ERC2 and ERC3 cells as described by Webb, *et al.* *Mol. Endocrinol.*, 6:157-167 (1993). It will be appreciated that the invention is not limited to practice in mammalian cells and may be practiced, for example in yeast and insect cells, transfected with the appropriate genes and recombinant constructs.

A) Cells naturally expressing two or more receptor types.

10 Many cells that express a second transcription factor receptor in addition to the estrogen receptor (ER) are well known to those of skill in the art. Thus, for example, in the uterus there is evidence that ER and glucocorticoid receptors (GR) co-exist in the endometrium (Prodi *et al.* (1979) *Tumor*. 65: 241-253). In the brain, maps of ER and GR immunoreactivity and mRNA localization suggest co-localization in certain
15 cerebral nuclei such as the paraventricular nucleus of the hypothalamus, the hypothalamic arcuate nucleus, and the central nucleus of the amygdala (Fuxe *et al.* (1985) *Endocrinol.*, 118: 1803-1812; Simerly *et al.* (1990) *J. Comp. Neurol.* 294: 76-95). In bone, ER and have been found in cultured osteoblast-like cells (Liesegang *et al.* (1994) *J. Andrology*, 14: 194-199). ER has also been demonstrated in osteoclasts (Oursler *et al.* (1994) *Proc.*
20 *Natl. Acad. Sci., USA*, 91: 5227-5231) and data suggest that the glucocorticoid dexamethasone (Dex) regulates metabolism in these cells (Wong (1979) *J. Biol. Chem.*, 254: 6337-6340) raising the possibility that osteoclasts contain functional GR as well. In addition, numerous tumor cell lines have been demonstrated to have both ER and GR (Ewing *et al.* (1989) *Int. J. Cancer.*, 44: 744-752.

25 B) Cells recombinantly modified to express two or more receptor types.

Cells normally lacking the ER α or ER β or other transcription factor cognate receptors can be recombinantly modified to express one or more of the desired receptors. Typically this involves transfecting the cell with an expression cassette comprising a nucleic acid encoding the receptor of interest and culturing the cell under
30 conditions where the receptor is expressed (*e.g.*, in the presence of an appropriate inducer

0 if the promoter regulating expression of the receptor is inducible). Typically, the cassette is selected to provide constitutive expression of the receptor.

A cell that naturally expresses one receptor need only be modified to express the second receptor. However, if the cell expresses neither receptor, it may be transfected with expression cassettes expressing both receptors. Even where a cell
5 naturally expresses one or both receptors, it may be recombinantly modified to express those receptors at a higher level (*e.g.*, by introducing expression cassettes encoding the receptor(s) whose expression level it is desired to increase).

The cells need not contain "native" receptors, but may be modified to provide truncated or chimeric receptors to provide increased affinity and/or sensitivity of
10 the assay. Thus, for example, Berry, *et al.* (1990), *EMBO J.*, 9: 2811-2818, describe the production of cells containing truncated or chimeric ER receptors.

Methods of modifying cells to express particular receptors are well known to those of skill in the art. Thus, for example, cells modified to express high levels of estrogen receptor are described by Kushner *et al.* (1990), *Mol. Endocrinol.*, 4:1465-1473.
15 *See also* Hirst *et al.* (1990) *Mol. Endocrinol.*, 4: 162-170). Transfection of cells to express ER α is described below, in the Examples, and in USSN 08/410,807. Transfection of cells to express ER β is described herein, and transfection of cells to express glucocorticoid receptors (GR), progestin receptors (PR), and other receptors is described in copending USSN 60/043,059.

20 C) Cells Containing AP1 proteins.

In assays that involve screening for transcription factor receptor mediated activation or inactivation of transcription at AP1, the cells preferably contain one or more AP1 proteins (the Jun or Fos proteins or other members of that protein family, *see* Bohmaan, *et al.* (1987) *Science*, 238: 1386-1392) in addition to the transcription factor
25 receptor(s).

The cells can naturally express the AP1 protein(s) or they can be modified (*e.g.*, by transfection with a suitable expression cassette) to express a heterologous AP1 protein. Methods of expressing AP1 proteins are well known to those of skill in the art (see, *e.g.*, Turner *et al.* (1989) *Science*, 243:1689-1694 and Cohen *et al.* (1989) *Genes*

0 & Dev., 3: 173-184, and Example 1). Cells that naturally express one or more AP1 proteins may still be so modified to increase intracellular jun and/or fos levels.

III) Expression of Nuclear Transcription Factor Receptors.

As explained above the assays of this invention utilize cells containing one or more nuclear transcription factor receptors (*e.g.*, ER α , ER β , GR, PR, *etc.*) an estrogen
5 receptor and a receptor for a nuclear transcription factor (typically a transcription factor other than estrogen). The factor can be one that is expressed endogenously by the cell or, alternatively, the cell can be modified (*e.g.*, a recombinant cell) so that it expresses the receptor.

A) Estrogen Receptor Alpha (ER α)

10 An estrogen receptor, as used herein, includes an estrogen receptor alpha (ER α) in its native (naturally occurring) form as well as modified estrogen receptors. Numerous modifications of estrogen receptors are known to those of skill in the art. These include, but are not limited to VP16-ER, V-ER, a chimeric receptor comprising the strong VP16 transcriptional activation domain linked to the amino terminus of the ER, V-
15 ER in which the ER DNA binding domain (DBD) is deleted, H11 an ER lacking the DNA binding domain, and the like (*see e.g.*, Kumar *et al.*, *Cell*, 51: 941-951 (1987) and Elliston *et al.* (1990) *J Biol Chem* 265:11517-21).

Means of recombinantly expressing the estrogen receptor alpha (ER α) are well known to those of skill in the art (*see, e.g.*, USSN 08/410,807 and Webb *et al.* (1995)
20 *Mol. Endocrinol.*, 9: 443-456).

B) Estrogen Receptor Beta (ER β).

Estrogen receptor beta (ER β) is a second estrogen receptor (ER) cloned from a rat prostate cDNA library (Kuiper *et al.* (1996) *Proc. Natl. Acad. Sci. USA*, 93: 5925-5930). Subsequently the human (Mosselman *et al.* (1996) *FEBS Lett.*, 392: 49-53)
25 and mouse (Tremblay *et al.* (1997) *Mol. Endocrinol.*, 11: 353-365) homologs were cloned. Accordingly, the original estrogen receptor (ER) has been renamed ER α (Kuiper *et al.* (1996) *supra.*).

Using the known sequence information one of skill in the art can routinely construct vectors that express an ER β when transfected into a suitable host cell. Detailed

0 protocols for the preparation of an ER β vector can be found in Kuiper *et al.* (1996) *Proc. Natl. Acad. Sci. USA*, 93: 5925-5930 and in WO 97/09348.

It will be appreciated that exist a number of different estrogen beta receptors comprising various splice variants, mutations, and so forth. It will be appreciated that ER β as used herein is intended to include all ER β variants. However, in
5 a preferred embodiment, the ER β variants used in this invention correspond to the so called "intermediate length" ER β variants such as those described in WO 97/09348. Particularly preferred ER β variants are shown in sequence listings 3, 4, and 5 herein which correspond to figures 1 and 13A and 13B of WO 97/09348,

C) Nuclear transcription factor ligand and cognate receptor

10 As indicated above, in addition to the estrogen receptor (ER α and/or ER β), the cells can contain a cognate receptor for a nuclear transcription factor ligand whose interaction (preferably a cognate receptor other than an estrogen receptor). As used herein, the term "cognate receptor" refers to a receptor of the type that is typically bound by the transcription factor ligand in question. Thus, the cognate receptor for an
15 estrogen is an estrogen receptor, the cognate receptor for a glucocorticoid is a glucocorticoid receptor, the receptor for a progestin is a progestin receptor, and so forth. As with the estrogen receptor, the cognate receptor includes the native (naturally occurring) form as well as modified receptors.

Natural and modified cognate receptors for nuclear transcription factor
20 ligands, particularly for steroid nuclear transcription factors, are well known to those of skill in the art. These include, but are not limited to the glucocorticoid receptors, the progestin receptors (*e.g.*, PR-A, PR-B (*see, e.g.*, Law *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84: 2877-2881; Wei *et al.* (1988) *Mol. Endo.* 2: 62-72; and Kushner *et al.* (1990) *Mol. Endocrinol.* 4:1465-1473), vitamin D receptors, mineralcorticoid receptors,
25 androgen receptors, and thyroid hormone receptors (*see*, Mangelsdorf (1995) *Cell*, 83: 835-839).

IV. ERE and AP1 Reporter Constructs

The cells of this invention preferably contain (*e.g.*, are transfected with) nucleic acid constructs comprising one or more reporter genes under the control of a
30 response element (either the AP1 site or estrogen response element (ERE)). Where two

0 different response elements are monitored in a single cell, two different reporter genes are used. Thus, for example, one gene can reports transcription induced by the classical estrogen response system (ERE), while the other gene reports transcription induced by the indirect (AP1) estrogen response. The two reporter genes and response elements are typically placed in separate cells, but the methods can also be used with both constructs
5 in the same cell.

A) AP1/Reporter construct.

In one embodiment the methods of this invention involve providing a cell containing an estrogen receptor (ER α or ER β), and a promoter comprising an AP1 site that regulates expression of a reporter gene (also referred to herein as the reporter gene
10 for the indirect estrogen response pathway (*see, e.g.*, USSN 08/410,807 and Webb *et al* (1995) *Mol. Endocrinol.*, 9: 443-456).

The reporter gene for the indirect estrogen response pathway contains an AP1 site preferably upstream of the target promoter and capable of regulating (*i.e.*, operably linked to) that promoter. AP1 site are sites that are bound by AP1 (the Jun and
15 Fos proteins) or other members of that protein family. In a preferred embodiment, the consensus AP1 site (or AP1 response element) is TGA(C/G)TCA (SEQ ID NO: 1).

One of skill would recognize that the particular AP1 site used is not a critical aspect of the invention. Any sequence capable of being bound by AP1 or members of that family and regulating a promoter is suitable. This would include promoters which
20 encompass a naturally occurring AP1 site. Typical promoters include, but are not restricted to metalloprotease genes such as stromelysin, gelatinase, matrilysin, and the human collagenase gene.

Alternatively promoters may be constructed which contain a non-naturally occurring AP1, or related, binding site. This facilitates the creation of reporter gene
25 systems that are not typically found under the control of AP1. In addition, promoters may be constructed which contain multiple copies of the AP1 site thereby increasing the sensitivity or possibly modulating the response the reporter gene system.

B) ERE/Reporter Construct

The methods of this invention can also involve providing a cell containing
30 a promoter comprising an estrogen response element that regulates expression of a

0 reporter gene (also referred to herein as the reporter gene for the direct or classical estrogen response pathway (see, e.g., U.S.S.N. 08/410,807 and Webb, *et al.* (1995) *Mol. Endo.*, 9: 443-456). This permits detection of the "direct" (classical) estrogen response and evaluation of the interaction or modulation of the classical response by the nuclear transcription factor ligand.

5 Typically, the estrogen response element (ERE) is upstream of the target promoter and capable of regulating that promoter. In a preferred embodiment the ERE may be the consensus estrogen response element AGGTCACAGTGACCT (SEQ ID NO: 2) from the *Xenopus* vitellogenin A2 gene. The particular ERE used in the cell is not a critical aspect of the invention and the present invention is not limited to the use of any
10 one particular ERE. Suitable EREs are well known to those of skill. For instance, other sources of naturally occurring EREs include the vitellogenin B2 gene, the chicken ovalbumin gene, and the PS2 gene. Alternatively, non-naturally occurring EREs may be inserted into particular promoters. The consensus ERE from the *Xenopus* vitellogenin A2 gene is widely used for this purpose, but other EREs may be used as well.

15 C) Reporter Gene(s)

The present invention is not limited to a particular reporter gene. Any gene that expresses an easily assayable product will provide a suitable indicator for the present assay. Suitable reporter genes are well known to those of skill in the art. Examples of reporter genes include, but are not limited to CAT (chloramphenicol acetyl
20 transferase) (Alton and Vapnek (1979) *Nature* 282: 864-869), luciferase, and other enzyme detection systems, such as beta-galactosidase; firefly luciferase (deWet *et al.* (1987) *Mol. Cell. Biol.* 7:725-737); bacterial luciferase (Engelbrecht *et al.* (1984) *Proc. Natl. Acad. Sci., USA*, 1: 4154-4158; Baldwin *et al.* (1984) *Biochemistry* 23:3663-3667); alkaline phosphatase (Toh *et al.* (1989) *Eur. J. Biochem.* 182: 231-238; Hall *et al.* (1983)
25 *J. Mol. Appl. Gen.* 2: 101), and green fluorescent protein.

One of skill will recognize that various recombinant constructs comprising the AP-1 site can be used in combination with any promoter and reporter gene compatible with the cell being used. The promoter will preferably be one susceptible to regulation by the AP1 site.

30 D) Construction of the Promoter/Reporter Expression Cassette.

0 The promoter/reporter expression cassettes and, other expression cassettes (constructs) described herein, can be constructed according to ordinary methods well known to those of skill in the art. Construction of these cassettes is variously exemplified in Example 1, in USSN 08/410,807, in Webb *et al.* (1995) *Mol. Endo.* 9: 443-456, and in other references cited herein.

5 The constructs can all be created using standard amplification and cloning methodologies well known to those of skill in the art. Examples of these techniques and instructions sufficient to direct persons of skill through many cloning exercises are found in Berger and Kimmel, *Guide to Molecular Cloning Techniques: Methods in Enzymology*, 152 Academic Press, Inc., San Diego, CA; Sambrook *et al.* (1989) *Molecular Cloning -*
10 *A Laboratory Manual (2nd ed.)* Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor Press, NY,; Current Protocols in Molecular Biology, Ausubel *et al.*, eds., *Current Protocols*, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1994 Supplement) (Ausubel); Cashion *et al.*, U.S. Patent No. 5,017,478; and Carr, European Patent No. 0,246,864. Examples of techniques sufficient to direct persons
15 of skill through in vitro amplification methods are found in Berger *supra.*, Sambrook *supra.*, and Ausubel *supra.*, as well as Mullis *et al.*, (1987) U.S. Patent No. 4,683,202; Innis *et al.* (1990) *PCR Protocols A Guide to Methods and Applications*, Academic Press Inc. San Diego, CA; Arnheim & Levinson (October 1, 1990) *C&EN* 36-47; *The Journal Of NIH Research* (1991) 3: 81-94; Kwoh *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:
20 1173; Guatelli *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87, 1874; Lomell *et al.* (1989) *J. Clin. Chem.*, 35: 1826; Landegren *et al.*, (1988) *Science*, 241: 1077-1080; Van Brunt (1990) *Biotechnology*, 8: 291-294; Wu and Wallace, (1989) *Gene*, 4: 560; and Barringer *et al.* (1990) *Gene*, 89: 117.

V. ER β -mediated Activation through tethered coactivators.

25 In still another embodiment, ER β can mediate gene activation through virtually any response element using a tethered transcription factor coactivator strategy. The methods involve contacting a nucleic acid that includes the gene of interest operably linked to a response element with a tethered coactivator. The tethered coactivator is composed of a polypeptide that comprises an activation function derived from a
30 transcriptional coactivator, and a DNA binding moiety that is capable of specifically

0 binding to the response element. The tethered coactivator is contacted with an activated transcription factor polypeptide (*e.g.*, ER β) that includes an activation function derived from a transcription factor. The contacting of the tethered coactivator with the activated transcription factor polypeptide stimulates expression of the gene. The transcription factor can be, for example, a nuclear hormone receptor such as the estrogen receptor or
5 the estrogen receptor beta, or an AP1 transcription factor, however, in a preferred embodiment, the transcription factor is ER β . Detailed protocols for the tethered transcription factor activation strategy are provided in copending USSN 60/043,059.

VI. Detection of the reporter genes.

Detection of the reporter genes of this invention is by standard methods
10 well known to those of skill in the art. Where the reporter gene is detected through its enzymatic activity this typically involves providing the enzyme with its appropriate substrate and detecting the reaction product (*e.g.*, light produced by luciferase). The detection may involve simply detecting presence or absence of reporter gene produce, or alternatively, detection may involve quantification of the level of expression of reporter
15 gene products. The quantification can be absolute quantification, or alternatively, can be comparative *e.g.*, with respect to the expression levels of one or more "housekeeping" genes. Methods of quantifying the expression levels of particular reporter genes are well known to those of skill in the art. It will be appreciated that such detection can be performed "manually" or may be automated *e.g.*, as in a high-throughput screening
20 system.

High throughput assays for the presence, absence, or quantification of gene expression (*e.g.*, via the detection of the transcribed nucleic acid (mRNA) or the detection of gene expression (protein product)) are well known to those of skill in the art. Thus, for example, U.S. Patent 5,559,410 discloses high throughput screening methods for proteins,
25 U.S. Patent 5,585,639 discloses high throughput screening methods for nucleic acid binding (*i.e.*, in arrays), while U.S. Patents 5,576,220 and 5,541,061 disclose high throughput methods of screening for ligand/antibody binding.

In addition, high throughput screening systems are commercially available (see, *e.g.*, Zymark Corp., Hopkinton, MA; Air Technical Industries, Mentor, OH;
30 Beckman Instruments, Inc. Fullerton, CA; Precision Systems, Inc., Natick, MA, *etc.*).

- 0 These systems typically automate entire procedures including all sample and reagent pipetting, liquid dispensing, timed incubations, and final readings of the microplate in detector(s) appropriate for the assay. These configurable systems provide high throughput and rapid start up as well as a high degree of flexibility and customization.

Compounds to be Screened.

- 5 It will be appreciated that virtually any compound can be screened by the methods of this invention. Such compounds include, but are not limited to known or suspected estrogens or antiestrogens including environmental estrogens or environmental antiestrogens as described above.

- 10 It will be appreciated that compounds are expected to show the most estrogenic or antiestrogenic activity if they are capable of penetrating to the nucleus of a cell and binding to a transcription factor receptor (*e.g.*, ER α or ER β). Such compounds are often lipophilic or capable of entering cells passively through pores or gates, through active transport, or through endocytosis. Particularly preferred compounds include, but are not limited to, steroid compounds or steroid analogs.

15 VIII. Assay Kits

- In another embodiment, this invention provides kits for the practice of the methods of this invention. The kits preferably include one or more containers containing the cells described herein for the practice of the assays of this invention. Thus, for example, the cells may include, but are not limited to, cells containing an estrogen receptor β (ER β), AP1 protein(s), and a construct comprising a promoter comprising an AP1 site which regulates expression of a first reporter gene, or such cells additionally containing a receptor for a nuclear transcription factor ligand other than estrogen. The AP1/reporter gene and the ERE/reporter gene constructs can be in separate cells or together in the same cell. The cells may additionally express high levels of AP1 proteins such as fos and/or jun. Alternatively, or in addition, the kits can contain the AP1/reporter gene and/or the ERE/reporter gene constructs described herein and/or the ER α , ER β , or other nuclear transcription factor receptor vectors. The kits may optionally contain any of the buffers, reagents, culture media, culture plates, reporter gene detection reagents, and so forth that are useful for the practice of the methods of this invention.
- 20
- 25

0 In addition, the kits may include instructional materials containing
directions (*i.e.*, protocols) for the practice of the assay methods of this invention. While
the instructional materials typically comprise written or printed materials they are not
limited to such. Any medium capable of storing such instructions and communicating
them to an end user is contemplated by this invention. Such media include, but are not
5 limited to electronic storage media (*e.g.*, magnetic discs, tapes, cartridges, chips), optical
media (*e.g.*, CD ROM), and the like. Such media may include addresses to internet sites
that provide such instructional materials.

EXAMPLES

The following examples are offered to illustrate, but not to limit the present
10 invention.

Example 1

Comparison of the Transactivation Properties of ER α and ER β

This example describes the investigation of the transactivation properties
of ER α and ER β with a panel of five ER ligands with the use of a reporter gene under the
15 control of either a classical ERE or an AP1 element. The results presented herein show
that ER α and ER β respond differently to certain ligands at an AP1 element suggesting
different regulatory functions for the two ER subtypes.

Screening Methods

20 The transactivation properties of ER α and ER β were compared with a
panel of five estrogen receptor (ER) ligands using a reporter gene under the control of
either a classical estrogen response element (ERE) or an AP1 element. The ERE and AP1
driven luciferase reporter plasmids (EREII-LucGl45 and Δ coll78, respectively) and the
ER α expression plasmid (pSG5-HEO) were used as described in Webb *et al.* (1995) *Mol.*
25 *Endocrinol.*, 9: 443-456, and in USSN 08/410,807 now issued as U.S. Patent __, __, __.

The rat ER β expression vector has been previously described (Kuiper *et al.* (1996) *Proc. Natl. Acad. Sci. USA*, 93: 5925-5930). The full-length human ER β
cDNA which was isolated from an ovarian cDNA library and found to be identical to the
30 previously reported partial cDNA clone (Mosselman *et al.* (1996) *FEBS Lett.*, 392: 49-

0 53) was cloned into the pCMV5 eukaryotic expression vector and the resulting ER β expression vector was used for these experiments (*see, Kuiper et al. (1996) Proc. Natl. Acad. Sci. USA, 93: 5925-5930*). The ligands used to compare ER α and ER β transactivation properties included the estrogens β -estradiol (E $_2$) and diethylstilbestrol (DES) and the antiestrogens Imperial Chemical Industries (ICI) 164384, tamoxifen, and
5 raloxifene. Raloxifene was synthesized according to published procedure (*Jones et al. (1984) J. Med. Chem., 27: 1057*). Structure and purity were verified by ^1H nuclear magnetic resonance (NMR), ^{13}C NMR, ultraviolet thin layer chromatography, and high resolution mass spectrometry. ICI 164384 was obtained from a private source and the other compounds were obtained from commercial sources.

10 The experiments were conducted by transfecting HeLa cells with either an ER α or ER β expression plasmid along with a reporter plasmid that contained a luciferase gene under the transcriptional control of an estrogen response element (ERE).

Cells were grown in Nunc Delta Surface tissue culture plates to a density of not more than 5×10^4 per cm^2 . Cells were grown in 0.1 μm sterile filtered DME-F-12
15 Coon's Modified Medium (Sigma Cell Culture) with 15 mM Hepes, 0.438 g/L L-glutamine, 1.338 g/L NaHCO_3 , 10% Seru-Max 4 (an iron supplemented, formula fed newborn calf serum, Sigma Cell culture; from a lot tested for low estrogenic activity), 0.05 mg/mL Gentamycin, 100 mg/ml Streptomycin SO_4 , and 100 units/ml penicillin "G".

Ishikawa cells were grown in a medium containing 100 nM tamoxifen and
20 MCF-7 cells were grown in medium containing 10 nM estradiol.

For the transfection assays, cells were suspended 0.5 ml of electroporation buffer in 0.4 cm gap electroporation cuvettes (BioRad) at 10^6 to 2×10^6 cells per cuvette. The electroporation buffer was prepared as a solution of 500 ml phosphate buffered saline (PBS), 5 ml of 10% glucose, and 50 μL of Biobrene. Five μg of reporter plasmid and 6
25 μg of ER expression plasmid were added and the cuvette was agitated to facilitate mixing of the solution and homogeneous cell distribution in the cuvette. Cells were then immediately transfected by electroporation with a BioRad GenePulser electroporation apparatus at a potential of 0.25 kV and a capacitance of 960 μF . To the electroporation cuvettes was added 1 ML growth medium (described above).

0 The transfected cells for one experiment were pooled and carefully resuspended in growth medium at a density of 8×10^4 - 1.6×10^5 cells/mL. After a homogenous cell distribution was obtained by thorough mixing cells were plated on Nunc 6-well dishes at 2 mL per well. After 2 h of incubation hormones were added and the medium was mixed by gentle swirling. Cells were then incubated in the presence of
5 hormone for 40-48 hours.

Growth medium was removed from the wells, and the cells were washed with Mg^{2+} and Ca^{2+} free PBS, and then they were lysed chemically with 0.2 mL of 100 mM potassium phosphate buffer (pH 7.5) containing 0.2% Triton X-100 and 1 mM DTT). The plates were then frozen to -80°C , thawed and scraped with a rubber policeman to
10 loosen and break up cell fragments. The lysate was centrifuged in a microfuge for 2 min, 0.1 mL of the supernatant was combined with 0.3 mL luciferase assay solution, and the chemiluminescence was measured immediately for a period of 10 s.

The luciferase assay solution consisted of 25 nM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA, 15 mM potassium phosphate at pH 7.8, with the addition of DTT
15 to a final concentration of 1 mM, ATP to a final concentration of 2 mM and luciferin (Analytical Luminescence Laboratories) to a final concentration of 200 μM shortly before commencing the assay. Luminescence measurements were performed on a Monolight 1500 (Analytical Luminescence Laboratories). The relative light units reported here were adjusted to a scale of 100 for uniformity.

20 The data were collected using the HEO ER variant. HEO shows reduced transactivation response from the unliganded receptor compared with the wild-type ER resulting in clearer ligand-induced transactivation data. Each experiment with $ER\alpha$ was also checked with the wild-type ER (HEGO), and the general ligand induction trends were found to the same as those obtained with HEO. The only difference was that the ligand-
25 induced transactivation responses were lower with HEGO than with the control (no ligand added).

Transactivation experiments were performed with both rat and human $ER\beta$ and identical trends in ligand behavior and similar induction levels were seen with both $ER\beta$ s in HeLa cells. The data shown in Figure 2B and Figure 4 were obtained with the
30 rat $ER\beta$ expression plasmid.

0

Experiments and Results

The transactivation properties of ER α and ER β at a classical ERE in response to the estrogens E₂ and diethylstilbestrol (DES) and the antiestrogens Imperial Chemical Industries (ICI) 164384, tamoxifen, and raloxifene were first investigated. Both ER α (18) and ER β (Fig. 2) showed the same transactivation profiles with the panel of ligands. E₂ and DES stimulated luciferase production 10-fold over ICI 164384, raloxifene, tamoxifen, and the control (no ligand added). The antiestrogens blocked E₂ stimulation in ligand competition experiments.

Next, the ligand-induced transactivation behavior of ER α and ER β at an AP1 site was examined. With ER α , all five ligands stimulated luciferase transcription, including the antiestrogens ICI 164384, tamoxifen, and raloxifene (Fig. 3). This stimulation was dependent on transfected ER, as cells transfected with only the reporter plasmid showed no induction of reporter transcription. Of the five ligands, raloxifene induced transcription the least, showing twofold induction compared with the sixfold inductions typically seen with E₂ and tamoxifen. The raloxifene-induced transactivation was dose dependent with a concentration value required for one-half maximal activation (EC₅₀) of about 1 nM. In addition, raloxifene reduced the activation caused by E₂ in a dose-dependent manner to the amount observed with raloxifene alone, demonstrating that raloxifene induction is weaker than induction by E₂ and that raloxifene-induced transactivation results from binding to ER α . If E₂ is classified as a full activator of ER α at an AP1 element (ER α -AP1), then raloxifene functions as a partial activator and tamoxifen functions as a full activator.

In contrast to the results seen with ER α -AP1, a difference in the ligand activation profile of ER β at an AP1 element (ER β -AP1) was observed. In cells transfected with ER β , treatment with the estrogens E₂ and DES did not increase luciferase transcription over the control (no ligand added), whereas treatment with the antiestrogens ICI 164384, raloxifene, and tamoxifen increased luciferase transcription (Fig. 4A). This transcription activation required transfected ER β , as wells that were transfected with only the reporter plasmid did not show transcriptional activation by the antiestrogens. The transcriptional activation caused by raloxifene was dose dependent with an EC₅₀ value of

0 about 50 nM (Fig. 4B). In ligand competition experiments, both E_2 and DES were able to block the raloxifene induction, and both estrogen ligands were able to reduce raloxifene induction to the basal level of transcription in a dose-dependent manner with concentration values required for one-half maximal inhibition of 1 to 10 nM (Fig. 4C).

5 In a different ligand competition experiment, the inhibitory effect on transcription resulting from E_2 treatment could be overcome by higher concentrations of raloxifene in a dose-dependent manner (Fig. 4D). Thus, it appears that the pharmacology of ER ligands is reversed at an AP1 element with ER β ; with ER β -AP1, the antiestrogens act as transcription activators, and the estrogens act as transcription inhibitors.

10 It was next investigated whether the action of ER β -AP1 could be observed in cell lines derived from estrogen target tissues such as the uterus and breast. Transactivation assays for ER β -AP1 were performed in Ishikawa cells (a human uterine cell line) (Fig. 5A) and in MCF7 (Fig. 5B) and MDA453 (Fig. 5c) human breast cancer cells. (The human ER β was used for transactivation in these cells.) In each of these cell lines, the ligands acted the same as they did in the HeLa cells; the three antiestrogens
15 activated and the estrogens inhibited ER β -dependent transcription from an AP1 site (Fig. 5). No induction was seen with cells that were not transfected with the ER β expression plasmid, indicating that the antiestrogen induction required ER β . Antiestrogen induction in the breast cell lines was higher than that observed in HeLa cells. Transfected MCF7 cells treated with raloxifene gave a 20- to 80-fold transactivation response over the
20 control (no ligand added). In addition, raloxifene and ICI 164384 induced transcription more than tamoxifen in the breast cell lines (Fig. 5, B and C).

MCF7 cells did not appear to contain high concentrations of endogenous ER β mRNA (Kuiper *et al.* (1997) *Endocrinol.*, 138: 553); however, the results suggest that the additional transactivation machinery required for ER β -AP1 function is present in
25 these cells. With two of these target tissue cell lines, E_2 treatment reduced the amount of transcription to less than that seen with the control (no ligand added). In MDA453 (Fig. 5C) and Ishikawa cells (Fig. 5A), E_2 treatment resulted in a consistent 40 to 75% reduction of reporter transcription levels compared with the control. This effect was also observed in ligand competition experiments (Fig. 5, A and C); E_2 and DES blocked
30 raloxifene induction and reduced the amount of transcription to less than that seen for the

0 control. Thus, when ER β is bound by the estrogen hormone E₂ or the synthetic estrogen DES, it functions as a negative regulator of genes controlled by an ER-dependent AP1 element.

The ER is the only known member of the steroidal subfamily of nuclear receptors that has different subtypes (Mangeldorf *et al.* (1996) *Cell*, 83: 835-839).
5 Nuclear receptors that respond to nonsteroidal hormones that have different known subtypes include the thyroid receptor (TR α and TR β), the retinoic acid receptor (RAR α , RAR β , and RAR γ), and the retinoid X receptor (RXR α , RXR β , and RXR γ) (Mangeldorf *et al.* (1996) *Cell*, 83: 841-850). The results presented herein demonstrate that two
10 nuclear receptor subtypes can respond in opposite regulatory modes to the natural hormone from the same DNA response element. Moreover, the ligand-induced responses with ER β at an AP1 site provide an example of negative transcriptional regulation by the natural hormone and strong positive regulation by synthetic antiestrogens. (The genes for transforming growth factor and quinone reductase are ER-regulated genes controlled by promoters containing nonclassical EREs that are activated by antiestrogens. However,
15 the action of ER β at either of these promoters has not been reported. The action of ER α on the quinone reductase gene shows a similar ligand profile to that of ER β at an AP1 site; antiestrogens are transcription activators, and E₂ is a transcription inhibitor.

If signaling from ER-dependent AP1 elements occurs in estrogen target tissues, the finding herein that ER α and ER β respond differently to ligands at AP1 sites
20 reveals a potential control mechanism for transcriptional regulation of estrogen-responsive genes and adds a layer of complexity in analyzing the pharmacology of antiestrogen therapeutics. The role of E₂ complexed to ER β would be to turn off the transcription of these genes, whereas the antiestrogens raloxifene, tamoxifen, and ICI 164384 could override this blockade and activate gene transcription. It will be T helpful to search for
25 genes in estrogen target tissues that are transcriptionally regulated by ER β at an AP1 site and to characterize the phenotype of cells in which these genes are activated.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will
30 be suggested to persons skilled in the art and are to be included within the spirit and

- 0 purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA
- (ii) TITLE OF INVENTION: DIFFERENTIAL LIGAND ACTIVATION OF ESTROGEN RECEPTORS ERalpha AND ERbeta AT AP1 SITES
- (iii) NUMBER OF SEQUENCES: 6
- (iv) CORRESPONDENCE ADDRESS:
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 - (F) ZIP: 90017-2576
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (vii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: BERLINER, Robert
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- (ix) TELECOMMUNICATION INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 7 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (ix) FEATURE:
 - (A) NAME/KEY: -
 - (B) LOCATION: 1..7
 - (D) OTHER INFORMATION: /note= "AP1 response element"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TGASTCA

7

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 15 base pairs
 - (B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

(A) NAME/KEY: -
(B) LOCATION: 1..15
(D) OTHER INFORMATION: /note= "ERE from the Xenopus vitellogenin A2 gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AGGTCACAGT GACCT

15

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2568 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION: 424..1878
(D) OTHER INFORMATION: /note= "Amino acid sequence of a rat ERbeta"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGAATTCGG GGGAGCTGGC CCAGGGGGAG CGGCTGGTGC TGCCACTGGC ATCCCTAGGC	60
ACCCAGGTCT GCAATAAAGT CTGGCAGCCA CTGCATGGCT GAGCGACAAC CAGTGGCTGG	120
GAGTCCGGCT CTGTGGCTGA GGAAAGCACC TGTCTGCATT TAGAGAATGC AAAATAGAGA	180
ATGTTTACCT GCCAGTCATT ACATCTGAGT CCCATGAGTC TCTGAGAACA TAATGTCCAT	240
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AAACTCACCG TCGAGCCTTA GTTCCTGCT TCCTATAACT GTAGCCAGTC CATCCTACCC	360
CTGGAGCACG GCCCATCTA CATCCCTTCC TCCTACGTAG ACAACCGCCA TGAGTATTCA	420
GCT ATG ACA TTC TAC AGT CCT GCT GTG ATG AAC TAC AGT GTT CCC GGC	468
Met Thr Phe Tyr Ser Pro Ala Val Met Asn Tyr Ser Val Pro Gly	
1 5 10 15	
AGC ACC AGT AAC CTG GAC GGT GGG CCT GTC CGA CTG AGC ACA AGC CCA	516
Ser Thr Ser Asn Leu Asp Gly Gly Pro Val Arg Leu Ser Thr Ser Pro	
20 25 30	
AAT GTG CTA TGG CCA ACT TCT GGG CAC CTG TCT CCT TTA GCG ACC CAT	564
Asn Val Leu Trp Pro Thr Ser Gly His Leu Ser Pro Leu Ala Thr His	
35 40 45	
TGC CAA TCA TCG CTC CTC TAT GCA GAA CCT CAA AAG AGT CCT TGG TGT	612
Cys Gln Ser Ser Leu Leu Tyr Ala Glu Pro Gln Lys Ser Pro Trp Cys	
50 55 60	
GAA GCA AGA TCA CTA GAG CAC ACC TTA CCT GTA AAC AGA GAG ACA CTG	660
Glu Ala Arg Ser Leu Glu His Thr Leu Pro Val Asn Arg Glu Thr Leu	
65 70 75	
AAG AGG AAG CTT AGT GGG AGC AGT TGT GCC AGC CCT GTT ACT AGT CCA	708
Lys Arg Lys Leu Ser Gly Ser Ser Cys Ala Ser Pro Val Thr Ser Pro	
80 85 90 95	

AAC GCA AAG AGG GAT GCT CAC TTC TGC CCC GTC TGC AGC GAT TAT GCA Asn Ala Lys Arg Asp Ala His Phe Cys Pro Val Cys Ser Asp Tyr Ala 100 105 110	756
TCT GGG TAT CAT TAC GGC GTT TGG TCA TGT GAA GGA TGT AAG GCC TTT Ser Gly Tyr His Tyr Gly Val Trp Ser Cys Glu Gly Cys Lys Ala Phe 115 120 125	804
TTT AAA AGA AGC ATT CAA GGA CAT AAT GAT TAT ATC TGT CCA GCC ACG Phe Lys Arg Ser Ile Gln Gly His Asn Asp Tyr Ile Cys Pro Ala Thr 130 135 140	852
AAT CAG TGT ACC ATA GAC AAG AAC CGG CGT AAA AGC TGC CAG GCC TGC Asn Gln Cys Thr Ile Asp Lys Asn Arg Arg Lys Ser Cys Gln Ala Cys 145 150 155	900
CGA CTT CGC AAG TGT TAT GAA GTA GGA ATG GTC AAG TGT GGA TCC AGG Arg Leu Arg Lys Cys Tyr Glu Val Gly Met Val Lys Cys Gly Ser Arg 160 165 170 175	948
AGA GAA CGG TGT GGG TAC CGT ATA GTG CGG AGG CAG AGA AGT TCT AGC Arg Glu Arg Cys Gly Tyr Arg Ile Val Arg Arg Gln Arg Ser Ser Ser 180 185 190	996
GAG CAG GTA CAC TGC CTG AGC AAA GCC AAG AGA AAC GGT GGG CAT GCA Glu Gln Val His Cys Leu Ser Lys Ala Lys Arg Asn Gly Gly His Ala 195 200 205	1044
CCC CGG GTG AAG GAG CTA CTG CTG AGC ACC TTG AGT CCA GAG CAA CTG Pro Arg Val Lys Glu Leu Leu Ser Thr Leu Ser Pro Glu Gln Leu 210 215 220	1092
GTG CTC ACC CTC CTG GAA GCT GAA CCA CCC AAT GTG CTG GTG AGC CGT Val Leu Thr Leu Leu Glu Ala Glu Pro Pro Asn Val Leu Val Ser Arg 225 230 235	1140
CCC AGC ATG CCC TTC ACC GAG GCC TCC ATG ATG ATG TCC CTC ACT AAG Pro Ser Met Pro Phe Thr Glu Ala Ser Met Met Met Ser Leu Thr Lys 240 245 250 255	1188
CTG GCG GAC AAG GAA CTG GTG CAC ATG ATT GGC TGG GCC AAG AAA ATC Leu Ala Asp Lys Glu Leu Val His Met Ile Gly Trp Ala Lys Lys Ile 260 265 270	1236
CCT GGC TTT GTG GAG CTC AGC CTG TTG GAC CAA GTC CGG CTC TTA GAA Pro Gly Phe Val Glu Leu Ser Leu Leu Asp Gln Val Arg Leu Leu Glu 275 280 285	1284
AGC TGC TGG ATG GAG GTG CTA ATG GTG GGA CTG ATG TGG CGC TCC ATC Ser Cys Trp Met Glu Val Leu Met Val Gly Leu Met Trp Arg Ser Ile 290 295 300	1332
GAC CAC CCC GGC AAG CTC ATT TTC GCT CCC GAC CTC GTT CTG GAC AGG Asp His Pro Gly Lys Leu Ile Phe Ala Pro Asp Leu Val Leu Asp Arg 305 310 315	1380
GAT GAG GGG AAG TGC GTA GAA GGG ATT CTG GAA ATC TTT GAC ATG CTC Asp Glu Gly Lys Cys Val Glu Gly Ile Leu Glu Ile Phe Asp Met Leu 320 325 330 335	1428
CTG GCG ACG ACG TCA AGG TTC CGT GAG TTA AAA CTC CAG CAC AAG GAG Leu Ala Thr Thr Ser Arg Phe Arg Glu Leu Lys Leu Gln His Lys Glu 340 345 350	1476
TAT CTC TGT GTG AAG GCC ATG ATC CTC CTC AAC TCC AGT ATG TAC CCC Tyr Leu Cys Val Lys Ala Met Ile Leu Leu Asn Ser Ser Met Tyr Pro 355 360 365	1524
TTG GCT TCT GCA AAC CAG GAG GCA GAA AGT AGC CGG AAG CTG ACA CAC Leu Ala Ser Ala Asn Gln Glu Ala Glu Ser Ser Arg Lys Leu Thr His 370 375 380	1572
CTA CTG AAC GCG GTG ACA GAT GCC CTG GTC TGG GTG ATT GCG AAG AGT	1620

Leu Leu Asn Ala Val Thr Asp Ala Leu Val Trp Val Ile Ala Lys Ser 385 390 395	
GGT ATC TCC TCC CAG CAG CAG TCA GTC CGA CTG GCC AAC CTC CTG ATG Gly Ile Ser Ser Gln Gln Gln Ser Val Arg Leu Ala Asn Leu Leu Met 400 405 410 415	1668
CTT CTT TCT CAC GTC AGG CAC ATC AGT AAC AAG GGC ATG GAA CAT CTG Leu Leu Ser His Val Arg His Ile Ser Asn Lys Gly Met Glu His Leu 420 425 430	1716
CTC AGC ATG AAG TGC AAA AAT GTG GTC CCG GTG TAT GAC CTG CTG CTG Leu Ser Met Lys Cys Lys Asn Val Val Pro Val Tyr Asp Leu Leu Leu 435 440 445	1764
GAG ATG CTG AAT GCT CAC ACG CTT CGA GGG TAC AAG TCC TCA ATC TCG Glu Met Leu Asn Ala His Thr Leu Arg Gly Tyr Lys Ser Ser Ile Ser 450 455 460	1812
GGG TCT GAG TGC AGC TCA ACA GAG GAC AGT AAG AAC AAA GAG AGC TCC Gly Ser Glu Cys Ser Ser Thr Glu Asp Ser Lys Asn Lys Glu Ser Ser 465 470 475	1860
CAG AAC CTA CAG TCT CAG TGATGGCCAG GCCTGAGGCG GACAGACTAC Gln Asn Leu Gln Ser Gln 480 485	1908
AGAGATGGTC AAAAGTGGAA CATGTACCCT AGCATCTGGG GGTTCCTCTT AGGGCTGCCT	1968
TGGTTACGCA CCCCTTACCC ACACCTGCACT TCCCAGGAGT CAGGGTGGTT GTGTGGCGGT	2028
GTTCCTCATA CCAGGATGTA CCACCGAATG CCAAGTTCTA ACTTGATAG CCTTGAAGGC	2088
TCTCGGTGTA CTACTTTCT GTCTCCTTGC CCACTYGGAA ACATCTGAAA GGTTCGGAA	2148
CTAAAGGTCA AAGTCTGATT TGAAGGATT GTCCTTAGTC AGGAAAAGGA ATATGGCATG	2208
TGACACAGCT ATAAGAAATG GACTGTAGGA CTGTGTGGCC ATAAATCAA CCTTGGATG	2268
GCGTCTTCTA GACCACTTGA TTGTAGGATT GAAAACCACA TTGACAATCA GCTCATTTCTG	2328
CATTCTGCCC TCACGGGTCT GTGAGGACTC ATTAATGTCA TGGGTTATTC TATCAAAGAC	2388
CAGAAAGATA GTGCAAGCTT AGATGTACCT TGTTCCTCCT CCCAGACCT TGGGTTACAT	2448
CCTTAGAGCC TGCTTATTG GTCTGTCTGA ATGTGGTCAT TGTCATGGGT TAAGATTAA	2508
ATCTCTTTGT AATATTGGCT TCCTTGAAGC TATGTCATCT TTCTCTCTCT CCCGGAATTT	2568

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 485 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) FEATURE:

- (A) -
- (B) -

(D) OTHER INFORMATION: /note= "Amino acid sequence of a rat ERbeta"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met	Thr	Phe	Tyr	Ser	Pro	Ala	Val	Met	Asn	Tyr	Ser	Val	Pro	Gly	Ser
1				5					10					15	

42

Thr Ser Asn Leu Asp Gly Gly Pro Val Arg Leu Ser Thr Ser Pro Asn
 20 25 30
 Val Leu Trp Pro Thr Ser Gly His Leu Ser Pro Leu Ala Thr His Cys
 35 40 45
 Gln Ser Ser Leu Leu Tyr Ala Glu Pro Gln Lys Ser Pro Trp Cys Glu
 50 55 60
 Ala Arg Ser Leu Glu His Thr Leu Pro Val Asn Arg Glu Thr Leu Lys
 65 70 75 80
 Arg Lys Leu Ser Gly Ser Ser Cys Ala Ser Pro Val Thr Ser Pro Asn
 85 90 95
 Ala Lys Arg Asp Ala His Phe Cys Pro Val Cys Ser Asp Tyr Ala Ser
 100 105 110
 Gly Tyr His Tyr Gly Val Trp Ser Cys Glu Gly Cys Lys Ala Phe Phe
 115 120 125
 Lys Arg Ser Ile Gln Gly His Asn Asp Tyr Ile Cys Pro Ala Thr Asn
 130 135 140
 Gln Cys Thr Ile Asp Lys Asn Arg Arg Lys Ser Cys Gln Ala Cys Arg
 145 150 155 160
 Leu Arg Lys Cys Tyr Glu Val Gly Met Val Lys Cys Gly Ser Arg Arg
 165 170 175
 Glu Arg Cys Gly Tyr Arg Ile Val Arg Arg Gln Arg Ser Ser Ser Glu
 180 185 190
 Gln Val His Cys Leu Ser Lys Ala Lys Arg Asn Gly Gly His Ala Pro
 195 200 205
 Arg Val Lys Glu Leu Leu Leu Ser Thr Leu Ser Pro Glu Gln Leu Val
 210 215 220
 Leu Thr Leu Leu Glu Ala Glu Pro Pro Asn Val Leu Val Ser Arg Pro
 225 230 235 240
 Ser Met Pro Phe Thr Glu Ala Ser Met Met Met Ser Leu Thr Lys Leu
 245 250 255
 Ala Asp Lys Glu Leu Val His Met Ile Gly Trp Ala Lys Lys Ile Pro
 260 265 270
 Gly Phe Val Glu Leu Ser Leu Leu Asp Gln Val Arg Leu Leu Glu Ser
 275 280 285
 Cys Trp Met Glu Val Leu Met Val Gly Leu Met Trp Arg Ser Ile Asp
 290 295 300
 His Pro Gly Lys Leu Ile Phe Ala Pro Asp Leu Val Leu Asp Arg Asp
 305 310 315 320
 Glu Gly Lys Cys Val Glu Gly Ile Leu Glu Ile Phe Asp Met Leu Leu
 325 330 335
 Ala Thr Thr Ser Arg Phe Arg Glu Leu Lys Leu Gln His Lys Glu Tyr
 340 345 350
 Leu Cys Val Lys Ala Met Ile Leu Leu Asn Ser Ser Met Tyr Pro Leu
 355 360 365
 Ala Ser Ala Asn Gln Glu Ala Glu Ser Ser Arg Lys Leu Thr His Leu
 370 375 380
 Leu Asn Ala Val Thr Asp Ala Leu Val Trp Val Ile Ala Lys Ser Gly
 385 390 395 400
 Ile Ser Ser Gln Gln Gln Ser Val Arg Leu Ala Asn Leu Leu Met Leu

43

405 410 415
 Leu Ser His Val Arg His Ile Ser Asn Lys Gly Met Glu His Leu Leu
 420 425 430
 Ser Met Lys Cys Lys Asn Val Val Pro Val Tyr Asp Leu Leu Leu Glu
 435 440 445
 Met Leu Asn Ala His Thr Leu Arg Gly Tyr Lys Ser Ser Ile Ser Gly
 450 455 460
 Ser Glu Cys Ser Ser Thr Glu Asp Ser Lys Asn Lys Glu Ser Ser Gln
 465 470 475 480
 Asn Leu Gln Ser Gln
 485

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 485 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: unknown
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(ix) FEATURE:

- (A) -
 (B) LOCATION: 1..485
 (D) OTHER INFORMATION: /note= "Amino acid sequence of human ERbeta"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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 20 25 30
 Val Leu Trp Pro Thr Pro Gly His Leu Ser Pro Leu Val Val His Arg
 35 40 45
 Gln Leu Ser His Leu Tyr Ala Glu Pro Gln Lys Ser Pro Trp Cys Glu
 50 55 60
 Ala Arg Ser Leu Glu His Thr Leu Pro Val Asn Arg Glu Thr Leu Lys
 65 70 75 80
 Arg Lys Val Ser Gly Asn Arg Cys Ala Ser Pro Val Thr Gly Pro Gly
 85 90 95
 Ser Lys Arg Asp Ala His Phe Cys Ala Val Cys Ser Asp Tyr Ala Ser
 100 105 110
 Gly Tyr His Tyr Gly Val Trp Ser Cys Glu Gly Cys Lys Ala Phe Phe
 115 120 125
 Lys Arg Ser Ile Gln Gly His Asn Asp Tyr Ile Cys Pro Ala Thr Asn
 130 135 140
 Gln Cys Thr Ile Asp Lys Asn Arg Arg Lys Ser Cys Gln Ala Cys Arg
 145 150 155 160
 Leu Arg Lys Cys Tyr Glu Val Gly Met Val Lys Cys Gly Ser Arg Arg
 165 170 175
 Glu Arg Cys Gly Tyr Arg Leu Val Arg Arg Gln Arg Ser Ala Asp Glu
 180 185 190

44

Gln Leu His Cys Ala Gly Lys Ala Lys Arg Ser Gly Gly His Ala Pro
 195 200 205
 Arg Val Arg Glu Leu Leu Leu Asp Ala Leu Ser Pro Glu Gln Leu Val
 210 215 220
 Leu Thr Leu Leu Glu Ala Glu Pro Pro His Val Leu Ile Ser Arg Pro
 225 230 235 240
 Ser Ala Pro Phe Thr Glu Ala Ser Met Met Met Leu Ser Thr Lys Leu
 245 250 255
 Ala Asp Lys Glu Leu Val His Met Ile Ser Trp Ala Lys Lys Ile Pro
 260 265 270
 Gly Phe Val Glu Leu Ser Leu Phe Asp Gln Val Arg Leu Leu Glu Ser
 275 280 285
 Cys Trp Met Glu Val Leu Met Met Gly Leu Met Trp Arg Ser Ile Asp
 290 295 300
 His Pro Gly Lys Leu Ile Phe Ala Pro Asp Leu Val Leu Asp Arg Asp
 305 310 315 320
 Glu Gly Lys Cys Val Glu Gly Ile Leu Glu Ile Phe Asp Met Leu Leu
 325 330 335
 Ala Thr Thr Ser Arg Phe Arg Glu Leu Lys Leu Gln His Lys Glu Tyr
 340 345 350
 Leu Cys Val Lys Ala Met Ile Leu Leu Asn Ser Ser Met Tyr Pro Leu
 355 360 365
 Val Thr Ala Thr Gln Asp Ala Asp Ser Ser Arg Lys Leu Ala His Leu
 370 375 380
 Leu Asn Ala Val Thr Asp Ala Leu Val Trp Val Ile Ala Lys Ser Gly
 385 390 395 400
 Ile Ser Ser Gln Gln Gln Ser Met Arg Leu Ala Asn Leu Leu Met Leu
 405 410 415
 Leu Ser His Val Arg His Ala Ser Asn Lys Gly Met Glu His Leu Leu
 420 425 430
 Asn Met Lys Cys Lys Asn Val Val Pro Val Tyr Asp Leu Leu Leu Glu
 435 440 445
 Met Leu Asn Ala His Val Leu Arg Gly Cys Lys Ser Ser Ile Thr Gly
 450 455 460
 Ser Glu Cys Ser Pro Ala Glu Asp Ser Lys Ser Lys Glu Gly Ser Gln
 465 470 475 480
 Asn Leu Gln Ser Gln
 485

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 1460 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: -
- (B) LOCATION: 1..1460

(D) OTHER INFORMATION: /note= "DNA sequence of human ERbeta"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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ACCTTTCTCC TTTAGTGGTC CATGCCAGT TATCACATCT GTATGCGGA CCTCAAAGA	180
GTCCCTGGTG TGAAGCAAGA TCGCTAGAAC ACACCTTACC TGTAACAGA GAGACACTGA	240
AAAGGAAGGT TAGTGGGAAC CGTTCGCCA GCCCTGTTAC TGGTCCAGG TCAAAGAGG	300
ATGCTCACTT CTGCGCTGTC TGCAGCGATT ACGCATCGGG ATATCACTAT GGAGTCTGGT	360
CGTGTGAAGG ATGTAAGGCC TTTTAAAAA GAAGCAGGCA AGGACATAAT GATTATATT	420
GTCCAGTAC AAATCAGTGT ACAATCGATA AAAACCGGG CAAGAGCTGC CAGGCTGCC	480
GACTTCGGA GTGTTACGAA GTGGGAATGG TGAAGTGTGG CTCCCGGAGA GAGAGATGTG	540
GGTACCGCCT TGTGCGGAGA CAGAGAAGTG CCGACGAGCA GCTGCACTGT GCCGGCAAGG	600
CCAAGAGAAG TGGCGGCCAC GCGCCCCGAG TCGGGAGCT GCTGCTGGAC GCCCTGAGCC	660
CCGAGCAGCT AGTGCTCACC CTCCTGGAGG CTGAGCCGCC CCATGTGCTG ATCAGCCGCC	720
CCAGTGCGCC CTTACCGAG GCCTCCATGA TGATGTCCCT GACCAAGTTG GCCGACAAGG	780
AGTTGTACA CATGATCAGC TGGGCCAAGA AGATTCCCG CTTTGTGGAG CTCAGCCTGT	840
TCGACCAAGT GCGGCTCTTG GAGAGCTGTT GGATGGAGGT GTTAATGATG GGGETGATG	900
GGCGCTCAAT TGACCACCCC GGCAAGCTCA TCTTTGCTCC AGATCTTGT CTGGACAGGG	960
ATGAGGGGAA ATGCGTAGAA GGAATTCTGG AAATCTTTGA CATGCTCTG GCAACTACTT	1020
CAAGGTTTCG AGAGTTAAAA CTCCAACACA AAGAATATCT CTGTGTCAAG GCCATGATCC	1080
TGCTCAATTC CAGTATGTAC CCTCTGGTCA CAGCGACCCA GGATGCTGAC AGCAGCCGGA	1140
AGCTGGCTCA CTTGCTGAAC GCCGTGACCG ATGCTTTGGT TTGGGTGATT GCCAAGAGCG	1200
GCATCTCTC CCAGCAGCAA TCCATGCGCC TGGCTAACCT CCTGATGCTC CTGTCCCAG	1260
TCAGGCATGC GAGTAACAAG GGCATGGAAC ATCTGCTCAA CATGAAGTGC AAAAAATGTG	1320
TCCCAGTGTG TGACCTGCTG CTGGAGATGC TGAATGCCA CGTGCTTCGC GGGTGCAAGT	1380
CCTCCATCAC GGGGTCCGAG TGCAGCCCG CAGAGGACAG TAAAGCAA GAGGGCTCCC	1440
AGAACCTACA GTCTCAGTGA	1460

0

WHAT IS CLAIMED IS:

1. A method of screening a test compound for differential ER α -mediated and ER β -mediated activation at an AP1 site, said method comprising the steps of:
 - a) providing a first cell comprising an estrogen receptor β (ER β), an AP1 protein, and a construct comprising a promoter comprising an AP1 site which regulates
5 expression of a first reporter gene;
 - b) contacting said first cell with said test compound; and
 - c) comparing the expression of said first reporter gene with the ER α -mediated expression of a gene at an AP1 site.
2. The method of claim 1, wherein said first cell contains a heterologous
10 estrogen receptor beta (ER β).
3. The method of claim 1, wherein said ER β comprises an amino acid sequence of SEQ ID NO: 3 or SEQ ID NO: 4.
4. The method of claim 1, wherein said cell contains a heterologous AP1 protein.
- 15 5. The method of claim 1, wherein said reporter gene is selected from the group consisting of chloramphenicol acetyl transferase (CAT), luciferase, β -galactosidase (β -gal), alkaline phosphatase, horse radish peroxidase (HRP), growth hormone (GH), and green fluorescent protein (GFP).
6. The method of claim 5, wherein said reporter gene encodes a luciferase
20 or a green fluorescent protein (GFP).
7. The method of claim 1, wherein said test compound is a test compound known to have anti-estrogenic activity.
8. The method of claim 1, wherein said ER α -mediated expression of a gene at an AP1 site is determined by:
 - 25 d) providing a second cell comprising an estrogen receptor α (ER α), AP1 proteins, and a construct comprising a promoter comprising an AP1 site which regulates expression of a second reporter gene;
 - e) contacting said second cell with said test compound; and
 - f) detecting expression of said second reporter gene

0 9. The method of claim 8, wherein said standard estrogen response element is from the *Xenopus* vitellogenin A2 gene.

 10. The method of claim 8, wherein said second reporter gene and said first reporter gene are the same reporter genes.

5 11. The method of claim 8, wherein said first cell and said second cell are the same cell.

 12. A method of screening a test compound for the ability to activate or inhibit estrogen receptor β (ER β) mediated gene activation at an AP1 site, said method comprising the steps of:

10 a) providing a first cell comprising an estrogen receptor β (ER β), AP1 proteins, and a construct comprising a promoter comprising an AP1 site which regulates expression of a first reporter gene;

 b) contacting said first cell with said test compound; and

 c) detecting expression of said first reporter gene.

15 13. The method of claim 12, wherein said first cell contains a heterologous estrogen receptor β (ER β).

 14. The method of claim 12, wherein said ER β comprises the amino acid sequence of Seq ID No: 3 or Seq ID NO: 5.

 15. The method of claim 14, wherein said first cell contains a heterologous AP1 protein.

20 16. The method of claim 12, wherein said reporter gene is selected from the group consisting of chloramphenicol acetyl transferase (CAT), luciferase, β - galactosidase (β -gal), alkaline phosphatase, horse radish peroxidase (HRP), growth hormone (GH), and green fluorescent protein (GFP).

25 17. The method of claim 16, wherein said reporter gene encodes a luciferase or a green fluorescent protein (GFP).

 18. The method of claim 12, wherein said test compound is a test compound known to have anti-estrogenic activity.

 19. The method of claim 12, further comprising the steps of:

0 d) providing a second cell comprising an estrogen receptor α (ER α), AP1 proteins, and a construct comprising a promoter comprising an AP1 site which regulates expression of a second reporter gene;

e) contacting said second cell with said test compound; and

f) detecting expression of said second reporter gene.

5 20. The method of claim 12, further comprising the steps of:

d) providing a third cell comprising an estrogen receptor α (ER α), and a construct comprising a promoter comprising a standard estrogen response element (ERE) which regulates expression of a third reporter gene;

e) contacting said third cell with said test compound; and

10 f) detecting expression of said third reporter gene.

21. The method of claim 20, wherein said standard estrogen response element is from the *Xenopus* vitellogenin A2 gene.

22. The method of claim 12, further comprising the steps of:

15 d) providing a fourth cell comprising an estrogen receptor β (ER β), and a construct comprising a promoter comprising a standard estrogen response element (ERE) which regulates expression of a fourth reporter gene;

e) contacting said fourth cell with said test compound; and

f) detecting expression of said fourth reporter gene.

20 23. The method of claim 22, wherein said standard estrogen response element is from the *Xenopus* vitellogenin A2 gene.

24. The method of claim 20, wherein said first cell and said third cell are the same cell.

25. The method of claim 22, wherein said first cell and said fourth cell are the same cell.

25 26. The method of claim 12, further comprising contacting said first cell with a second compound, in addition to said test compound, wherein said second compound is known to activate transcription through estrogen receptor β (ER β) mediated gene activation at an AP1 site;

30 wherein said detecting comprises detecting test compound mediated decrease in said estrogen receptor β (ER β) mediated gene activation at an AP1 site.

0 27. The method of claim 26, wherein said detecting comprises comparing the expression of said first reporter gene in the presence of the test compound and the second compound with the expression of said first reporter gene in the presence of the second compound without the test compound.

5 28. The method of claim 26, wherein said second compound known to activate transcription through estrogen receptor β (ER β) mediated gene activation at an AP1 site is identified by a method comprising the steps of:

 a) providing a second cell comprising an estrogen receptor β (ER β), and AP1 protein, and a construct comprising a promoter comprising an AP1 site that regulates expression of a second reporter gene;

10 b) contacting said second cell with second compound; and

 c) detecting the expression of said second reporter gene, wherein an increase in expression of said second reporter gene produced by said compound indicates that said second compound activates transcription through ER β at said AP1 site.

15 29. The method of claim 12, further comprising contacting said first cell with a second compound, in addition to said test compound, wherein said second compound is known to inhibit transcription through estrogen receptor β (ER β) mediated activity at an AP1 site; and wherein said detecting comprises detecting test compound mediated increase in estrogen receptor β (ER β) mediated gene activation at an AP1 site.

20 30. The method of claim 29, wherein said detecting comprises comparing the expression of said first reporter gene in the presence of said second compound and said test compound with the expression of said first reporter gene in the presence of said second compound without said test compound.

25 31. The method of claim 29, wherein said second compound known to inhibit transcription through estrogen receptor β (ER β) mediated gene activation at an AP1 site is identified by a method comprising the steps of:

 a) providing a second cell comprising an estrogen receptor β (ER β), and AP1 protein, and a construct comprising a promoter comprising an AP1 site that regulates expression of a second reporter gene;

 b) contacting said second cell with second compound; and

0 c) detecting the expression of said second reporter gene, wherein a decrease in expression of said second reporter gene produced by said compound indicates that said second compound inhibits transcription through ER β at said AP1 site.

32. A cell comprising an estrogen receptor β (ER β), AP1 proteins, and a construct comprising a promoter comprising an AP1 site which regulates expression of
5 a first reporter gene.

33. The cell of claim 32, wherein said cell further contains a receptor for a nuclear transcription factor ligand other than estrogen.

34. The cell of claim 32, wherein said cell contains a heterologous estrogen receptor β (ER β).

10 35. The cell of claim 32, wherein said cell contains a heterologous AP1 protein.

36. The cell of claim 32, wherein said heterologous AP1 protein is c-jun.

37. The cell of claim 32, wherein said first reporter gene is selected from the group consisting of chloramphenicol acetyl transferase (CAT), luciferase, β - galactosidase (β -gal), alkaline phosphatase, horse radish peroxidase (HRP), growth
15 hormone (GH), and green fluorescent protein (GFP).

38. The cell of claim 37, wherein said reporter gene encodes a luciferase or a green fluorescent protein (GFP).

39. The cell of claim 38, wherein said cell further comprises a construct
20 comprising a promoter comprising a standard estrogen response element (ERE) which regulates expression of a second reporter gene.

40. The cell of claim 39, wherein said standard estrogen response element is from the *Xenopus* vitellogenin A2 gene.

41. The cell of claim 32, wherein said cell is a mammalian cell.

25 42. The cell of claim 41, wherein said cell is derived from breast tissue or from uterine tissue.

43. A method of screening a nuclear transcription factor ligand for the ability to modulate estrogen receptor β mediated activation or inactivation of transcription at an AP1 site, said method comprising the steps of:

0 a) providing a first cell containing an estrogen receptor β (ER β), an AP1 protein, a receptor for said nuclear transcription factor ligand, and a construct comprising a promoter comprising an AP1 site which regulates expression of a first reporter gene;

b) contacting said first cell with said transcription factor ligand and with a compound having ER β mediated activity at said AP1 site; and

5 c) detecting expression of said first reporter gene.

44. The method of claim 43, further comprising the steps of:

d) providing a second cell containing an estrogen receptor β (ER β), a receptor for said nuclear transcription factor ligand, and a construct comprising a promoter comprising an estrogen response element (ERE) that regulates expression of a

10 second reporter gene;

e) contacting said second cell with said transcription factor ligand and with said compound having AP-1 mediated estrogenic activity; and

f) detecting expression of said second reporter gene.

15 45. The method of claim 44, wherein said first cell and said second cell are the same cell.

46. The method of claim 43, further comprising the steps of:

d) providing a second cell containing a cognate receptor of said transcription factor ligand, and a promoter comprising a response element for said cognate receptor that regulates expression of a second reporter gene;

20 e) contacting said second cell with said transcription factor ligand and with said compound having compound having ER β mediated activity at said AP1 site; and

f) detecting expression of said second reporter gene.

47. The method of claim 46, wherein said first cell and said second cell are the same cell.

25 48. The method of claim 43, wherein said nuclear transcription factor ligand is selected from the group consisting of a glucocorticoid, a progestin, vitamin D, retinoic acid, a an androgen, a mineralcorticoid, and a prostaglandin.

49. The method of claim 46, wherein said cognate receptor is selected from the group consisting of an estrogen receptor, a glucocorticoid receptor, a progestin

0 PR-A receptor, and progestin PR-B receptor, androgen receptor, a mineralcorticoid receptor, and a prostaglandin receptor.

50. The method of claim 43, wherein said ER β comprises an amino acid sequence of Seq ID No: 3 or SEQ ID No: 5.

5 51. The method of claim 43, wherein said estrogen receptor ER β is heterologous to said cell.

52. The method of claim 43, wherein said receptor for said nuclear transcription factor ligand is heterologous to said cell.

53. The method of claim 43, wherein said cell expresses an AP1 protein from a heterologous DNA.

10 54. The method of claim 53, wherein said AP1 protein is c-jun.

55. The method of claim 43, wherein said nuclear transcription factor is a progestin; and said receptor for said nuclear transcription factor ligand is a progestin receptor.

15 56. The method of claim 43, wherein said nuclear transcription factor is a glucocorticoid and said receptor for said nuclear transcription factor ligand is a GR receptor.

0 57. A method of screening an agent for the ability to alter modulation of estrogen receptor β (ER β) activation or inactivation of transcription at an AP1 site by a nuclear transcription factor ligand, said method comprising the steps of:

 a) providing a first cell containing an estrogen receptor β (ER β), an AP1 protein, a receptor for said nuclear transcription factor ligand, and a promoter comprising
5 an AP1 site which regulates expression of a first reporter gene;

 b) contacting said first cell with said transcription factor ligand, with a compound having ER β mediated activity at an AP1 site, and with said agent; and

 c) detecting expression of said first reporter gene.

58. The method of claim 57, further comprising the steps of:

10 d) providing a second cell containing an estrogen receptor β (ER β), a receptor for said nuclear transcription factor ligand, and a promoter comprising an estrogen response element (ERE) that regulates expression of a second reporter gene;

 e) contacting said second cell with said transcription factor ligand and with said compound having AP-1 mediated estrogenic activity; and

15 f) detecting expression of said second reporter gene.

59. The method of claim 58, wherein said first cell and said second cell are the same cell.

60. The method of claim 57, wherein said nuclear transcription factor is selected from the group consisting of a glucocorticoid, a progestin, vitamin D, retinoic
20 acid, an androgen, a mineralcorticoid, a prostaglandin.

61. The method of claim 57, wherein said a receptor for said nuclear transcription factor ligand is selected from the group consisting of an estrogen receptor, a glucocorticoid receptor, a progestin PR-A receptor, progestin PR-B receptor, an androgen receptor, a mineralcorticoid receptor, and a prostaglandin receptor.

25 62. The method of claim 57, wherein said cell contains a heterologous estrogen receptor β (ER β).

63. The method of claim 57, wherein said cell expresses a heterologous receptor for said nuclear transcription factor ligand.

30 64. The method of claim 57, wherein said cell contains a heterologous AP1 protein.

- 0 65. The method of claim 64, wherein said AP1 protein is c-jun.
66. The method of claim 57, wherein said nuclear transcription factor is
a progestin; and said receptor for said nuclear transcription factor ligand is a progestin
receptor.
- 5 67. The method of claim 57, wherein said nuclear transcription factor is
a glucocorticoid and said receptor for said nuclear transcription factor ligand is a GR
receptor.
68. A kit for screening a compound for the ability to activate or inhibit
estrogen receptor β (ER β) mediated gene activation at an AP1 site, said kit comprising
a container containing a cell comprising an estrogen receptor β (ER β), an AP1 protein,
10 and a construct comprising a promoter comprising an AP1 site which regulates expression
of a first reporter gene.
69. The kit of claim 68, further comprising instruction materials containing
protocols for the practice of the assay methods of claims 1, 9, 10, 12, 16, or 18.
70. The kit of claim 68, wherein said cell further comprises a receptor for
15 a nuclear transcription factor ligand other than estrogen.
71. The kit of claim 68, further comprising instruction materials containing
protocols for the practice of the assay methods of claims 29, 30, 32, 42, or 43.

1/3

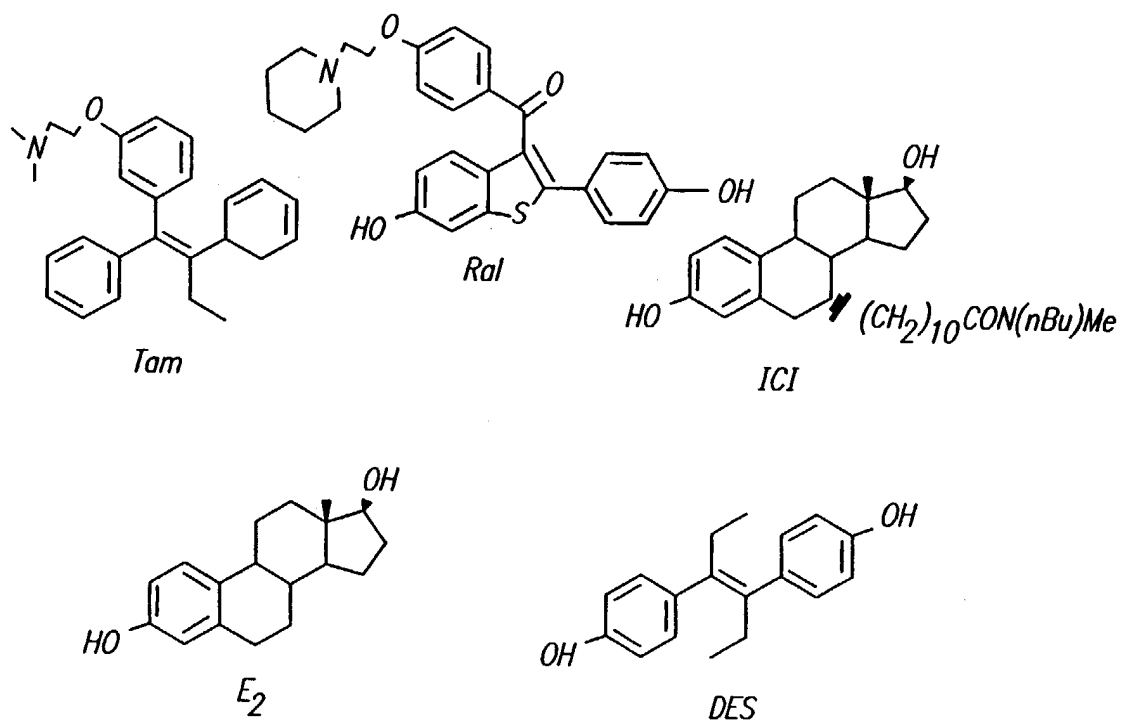


FIG. 1A

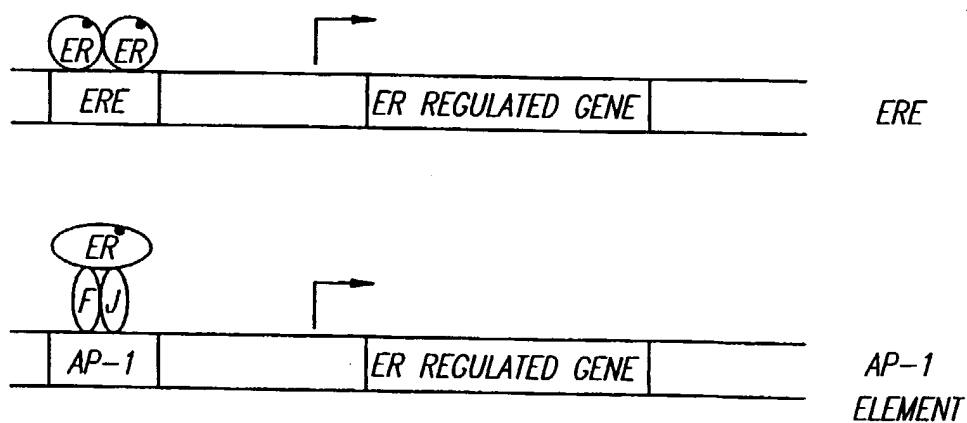


FIG. 1B

2/3

FIG. 2

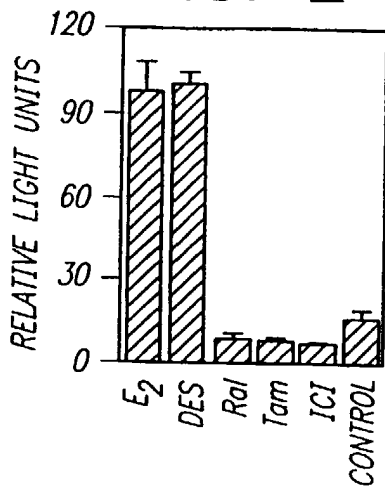


FIG. 3

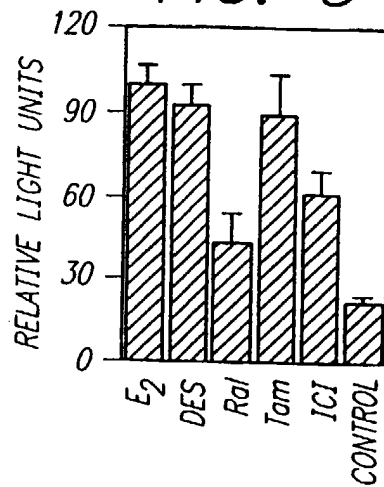


FIG. 4A

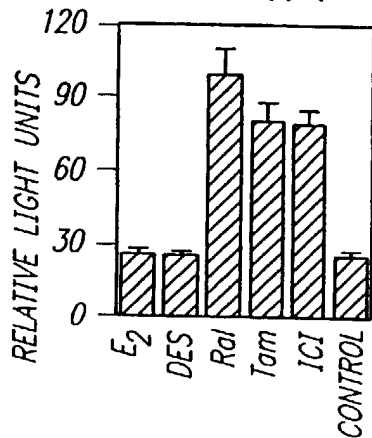


FIG. 4B

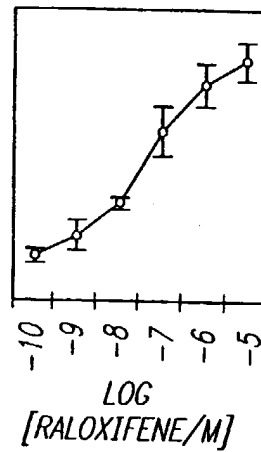


FIG. 4C

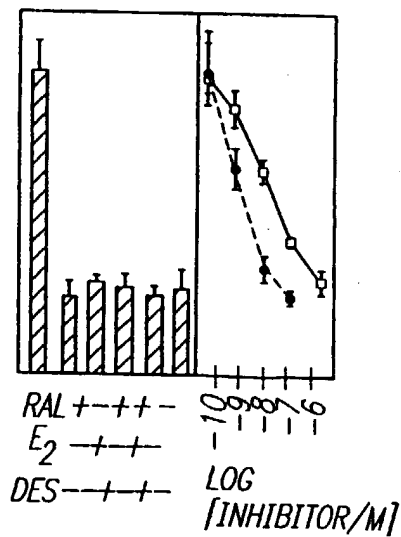
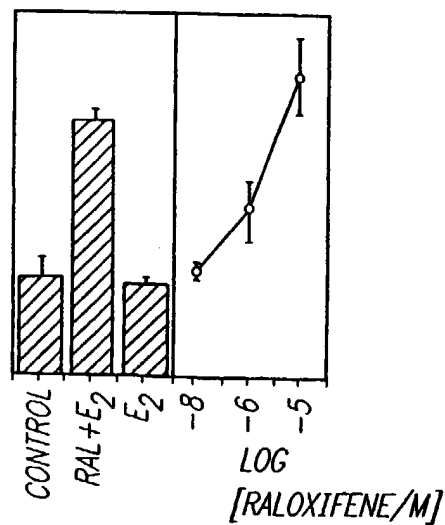


FIG. 4D



3/3

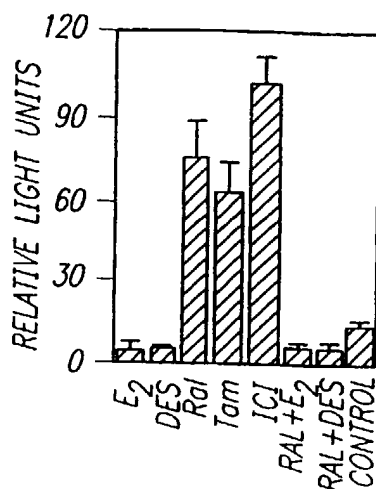


FIG. 5A

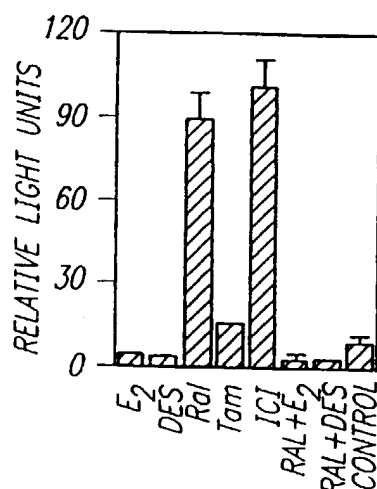


FIG. 5B

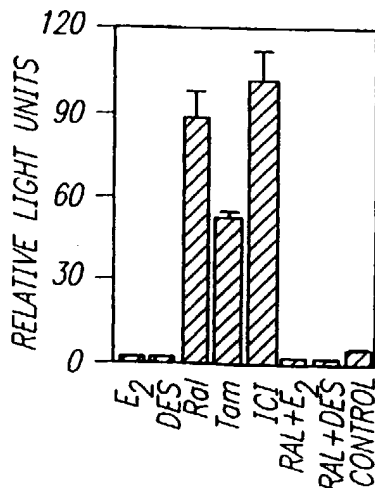


FIG. 5C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/18030

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 5/10, 15/09; G01N 33/53, 33/566

US CL : 435/6, 7.8, 69.1, 325

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.8, 69.1, 325

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y - A	WEBB et al. Tamoxifen activation of the estrogen receptor/AP-1 pathway: potential origin for the cell-specific estrogen-like effects of antiestrogens. April 1995, Vol. 9, No.4, pp. 443-456, see especially pages 443-444, 447, and Table 1.	1-47, 50-54 ----- 48-49, 55-56
Y - A	MOSSELMAN et al. ERbeta: identification and characterization of a novel human estrogen receptor. 19 August 1996, Vol. 392, No. 1, pp. 49-53, especially pages 49-50, Figures 1 and 3.	1-47, 50-54 ----- 48-49, 55-56

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

22 NOVEMBER 1998

Date of mailing of the international search report

30 DEC 1998

Name and mailing address of the ISA/US
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/18030

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, STN, BIOSCIENCE, CAPLUS, MEDLINE, BIOSIS, EMBASE

search terms: estrogen receptor, ap1, ER-alpha, ER-beta, reporter